Claims Not Listed

FEE TRANSMITTAL
For FY 2005

MAIL STOP APPEAL BRIEFS-PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27

Total Amount Of Payment ($) **500.00**

Complete If Known	
Application No.	08/479,997
Filing Date	June 7, 1995
First Named Inventor	Engelhardt et al
Examiner Name	Ardin H. Marchel, Ph.D
Art Unit	1631
Attorney Docket No.	Enz-5(D6)(C2)

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (identify): _____

☒ Deposit Account Deposit Account Number **05-1135** Deposit Account Name : **Enzo Biochem, Inc.**

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☒ Charge fee(s) indicated below.
☒ Charge any additional fee(s) or underpayments of fee(s) under 37 CFR 1.16 and 1.17
☒ Charge fee(s) indicated below, **except for the filing fee**
☒ Credit any overpayments.

FEE CALCULATION

1. BASIC FILING, SEARCH AND EXAMINATION FEES

	Filing Fees		Search Fees		Examination Fees		
Application Type	**Fee($)**	**Small Entity Fee ($)**	**Fee($)**	**Small Entity Fee ($)**	**Fee($)**	**Small Entity Fee ($)**	**Fees Paid ($)**
Utility	300.00	150.00	500.00	250.00	200.00	100.00	
Design	200.00	100.00	100.00	50.00	130.00	65.00	
Plant	200.00	100.00	300.00	150.00	160.00	80.00	
Reissue	300.00	150.00	500.00	250.00	600.00	300.00	
Provisional	200.00	100.00	0.00	0.00	0.00	0.00	

2. EXCESS CLAIMS FEES

For	**Number Present**	**Highest Number Paid For**	**Extra**	**Fees ($)**	**Small Entity Fee ($)**	**Fees Paid ($)**
Total Claims		20	0 x	50.00	25.00	
Independent Claims		3	0 x	200.00	100.00	
Multiple Dependent Claim				360.00	180.00	
Total Excess Claims Fees						

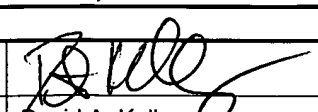
3. APPLICATION SIZE FEE (if the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	**Extra Sheets**	**No. of Each Additional 50 or Fraction Thereof**	**Fees ($)**	**Small Entity Fee ($)**	**Fees Paid ($)**
_____ - 100 =	_____ / 50 =	_____ (round up to a whole number) x	250.00	125.00	

4. OTHER FEE(S)

☐	Non-English Specification (no small entity discount)	☐	Utility Issue Fee (or Reissue) (including Publication Fee, if necessary)
☐	Surcharge - late filing fee or oath	☐	Design Issue Fee
☐	Surcharge - late provisional filing fee or cover sheet	☐	Plant Issue Fee
☐	_____ Month Extension of Time	☐	Petition to Commissioner
☐	Submission of Information Disclosure Statement	☐	Petition to Revive (Unavoidable)
☐	Notice of Appeal	☐	Petition to Revive (Unintentional)
☐	Request for Oral Hearing	☐	Petitions Related to Provisional Applications
☒	Filing Brief in Support of Appeal **$500**	☐	Recording Each Patent Assignment Per Property
☐	Filing Submission After Final Rejection	☐	Other (specify) _____

SUBMITTED BY

Signature		Registration No.	53,106	Telephone	202 955-1500
Typed or Printed Name	David A. Kelly			Date	April 4, 2005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Engelhardt et al.	
Serial No.:	08/479,997	Group Art Unit: 1631
Filed:	June 7, 1995	Examiner: Ardin H. Marchel, Ph.D
For:	**OLIGO- OR POLYNUCLEOTIDES, COMPRISING PHOSPHATE-MOIETY LABELED NUCLEOTIDES**	Atty. Dkt. No. Enz-5(D6)(C2)

APPEAL BRIEF UNDER 37 C.F.R. § 41.37

Commissioner for Patents
U.S. Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is an appeal pursuant to 35 U.S.C. § 134 from the Examiner's decision rejecting claims 956-961, 964-968, 970-976, 978-993, 996-1000, 1002-1009, 1011-1027, 1030-1034, 1036-1042, 1044-1056, 1062-1066, 1068-1075, and 1077-1087 as set forth in the final Office action mailed July 14, 2004.

This Appeal Brief sets forth below each item required by 37 C.F.R. § 41.37(c)(1). The Notice of Appeal was filed on September 3, 2004. Appellants have already paid for a five-month extension of time thereby extending the deadline for filing the Appeal Brief to Monday, April 4, 2005, as April 3, 2005 falls on a Sunday. Pursuant to 37 C.F.R. § 41.37 (e), Appellants believe a fee of $500.00 is required for the Appeal Brief. The Commissioner is hereby authorized to charge the undersigned's Deposit Account No. 05-1135 for any fees associated with the filing of this Appeal Brief.

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REQUIREMENTS OF 37 C.F.R. § 1.192(c)

I. Real party in interest.

The real party in interest of the application is Enzo Life Sciences, Inc., which is a subsidiary of Enzo Biochem, Inc. (hereinafter "Enzo"). Enzo Life Science, Inc. was formerly named Enzo Diagnostics, Inc. Enzo is the owner of the application by way of an assignment from the inventors, Engelhardt et al., of all rights, title, and interests.

II. Related appeals and interferences.

None.

III. Status of claims.

Claims 1-955, 962-963, 977, 994-995, 1001, 1010, 1028-1029, 1035, 1043, 1060-1061, 1067, 1076 and 1088-1227 stand cancelled without prejudice to the subject matter claimed therein.

Claims 956-961, 964-968, 970-976, 978-993, 996-1000, 1002-1009, 1011-1027, 1030-1034, 1036-1042, 1044-1056, 1062-1066, 1068-1075 and 1077-1087 stand finally rejected and are the subject of this appeal.

IV. Status of amendments.

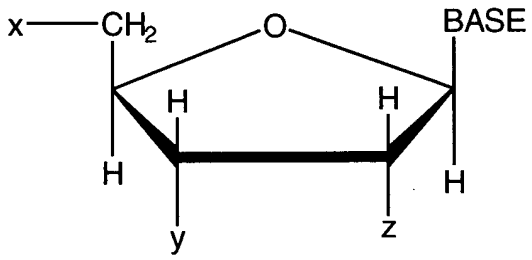
In a telephonic interview conducted on March 29, 2005 between Appellants' legal representatives and representatives from Group 1600 of the U.S. Patent and Trademark Office ("USPTO"), Appellants agreed to file a Supplemental Amendment substantially similar to their December 28, 2004 Amendment, except that Claims 956, 988, 1022 and 1054 would recite "and wherein said Sig comprises a non-polypeptide, ~~non-nucleotidyl,~~ non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polynucleotide or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof." In turn, the Examiner agreed to enter the amendment for purposes of appeal and noted that the amendment would overcome the rejection of the claims under 35 U.S.C. § 112, paragraphs 1 and 2. The Examiner indicated, however, that the rejection of the claims under 35 U.S.C. § 102(a) and (b) would remain as stated in the final Office action mailed July 14, 2004.

In an interview summary dated March 31, 2005, the Examiner stated that Appellants' Second Supplemental Amendment, filed on March 30, 2005 and incorporating those changes agreed to in the March 29 telephonic conference, would be entered for appeal.

V. Summary of claimed subject matter.

Independent claim 956 recites, "956. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising: (pg. 5, ll. 18 to pg. 6, ll. 2; pg. 25, ll. 22-34) at least one modified nucleotide or modified nucleotide analog having the formula Sig—PM—SM—BASE (pg. 94, ll. 21-28) wherein PM is a phosphate moiety, (pg. 96, ll. 1-12) SM is a furanosyl moiety (pg. 96, ll. 1-12) and BASE is a base moiety comprising a pyrimidine, a pyrimidine analog, a purine, a purine analog, a deazapurine or a deazapurine analog (pg. 90, ll. 28 to pg. 92, end; pg. 94, ll. 21 to pg. 95, end) wherein said analog can be attached to or coupled to or incorporated into DNA or RNA wherein said analog does not substantially interfere with double helix formation or nucleic acid hybridization (pg. 52, ll. 30 to pg. 53, ll. 4) said PM being attached to SM, (pg. 94, ll. 21-28) said BASE being attached to SM, (pg. 94, ll. 21-28) and said Sig being covalently attached to PM directly or through a chemical linkage, and wherein said Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM (pg. 94, ll. 21-28; pg. 97, ll. 22 to pg. 98, ll. 14) or when said modified nucleotide is incorporated into said oligo- or polynucleotide or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof (pg. 95, ll. 2-11) and wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing (pg. 82, ll. to pg. 84, ll. 10; pg. 96, ll. 22 to pg. 97, ll. 28)."

Independent claim 988 recites, "988. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising: (pg. 5, ll. 18 to pg. 6, ll. 2; pg. 25, ll. 22-34) at least one modified nucleotide or a modified nucleotide analog having the structural formula:



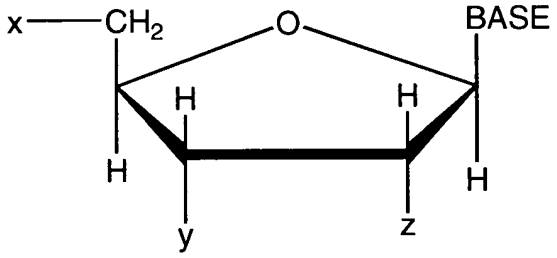
(pg. 2, ll. 25 to p.5, ll. 7; pg. 94, ll. 21-28) wherein BASE is a moiety comprising a pyrimidine, a pyrimidine analog, a purine, a purine analog, a deazapurine or a deazapurine analog, (pg. 90, ll. 28 to pg. 92, end; pg. 94, ll. 21 to pg. 95, end) wherein said analog can be attached to or coupled to or incorporated into DNA or RNA, wherein said analog does not substantially interfere with double helix formation or nucleic acid hybridization, (pg. 52, ll. 30 to pg. 53, ll. 4) and wherein said BASE is attached to the 1' position of the furanosyl ring from the N1 position when said BASE is a pyrimidine or a pyrimidine analog, or from the N9 position when said BASE is a purine, a purine analog, a deazapurine or a deazapurine analog; (pg. 8, ll. 21-27; pg. 93, ll. 15-18) wherein x comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate; wherein y comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate; wherein z comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate; and wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate comprising x, y, z, or a combination thereof, (pg. 3, ll. 1-32; pg. 9, ll. 4-14; pg. 12, ll. 6-25) and wherein said Sig comprises a non-polypeptide, non-radioactive label moiety which can

be directly or indirectly detected when attached to said phosphate (pg. 94, ll. 21-28; pg. 97, ll. 22 to pg. 98, ll. 14) or when said modified nucleotide is incorporated into said oligo- or polynucleotide or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, (pg. 95, ll. 2-11) wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing. (pg. 82, ll. to pg. 84, ll. 10; pg. 96, ll. 22 to pg. 97, ll. 28)."

Independent claim 1022 recites, "1022. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising: (pg. 5, ll. 18 to pg. 6, ll. 2; pg. 25, ll. 22-34) at least one modified nucleotide or a modified nucleotide analog having the formula Sig—PM—SM—BASE (pg. 94, ll. 21-28) wherein PM is a phosphate moiety, (pg. 96, ll. 1-12) SM is a furanosyl moiety (pg. 96, ll. 1-12) and BASE is a base moiety comprising a pyrimidine, a pyrimidine analog, a purine, a purine analog, a deazapurine or a deazapurine analog, (pg. 90, ll. 28 to pg. 92, end; pg. 94, ll. 21 to pg. 95, end) wherein said analog can be attached to or coupled to or incorporated into DNA or RNA, wherein said analog does not substantially interfere with double helix formation or nucleic acid hybridization, (pg. 52, ll. 30 to pg. 53, ll. 4) said PM is attached to SM, (pg. 94, ll. 21-28) said BASE is attached to SM, (pg. 94, ll. 21-28) said Sig is covalently attached to PM directly or via a chemical linkage, and wherein said Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM (pg. 94, ll. 21-28; pg. 97, ll. 22 to pg. 98, ll. 14) or when said modified nucleotide is incorporated into said oligo- or

polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, (pg. 95, ll. 2-11) provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide, (pg. 53, ll. 18-27; pg. 94, ll. 21 to pg. 95, ll. 11) and wherein said Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing. (pg. 82, ll. to pg. 84, ll. 10; pg. 96, ll. 22 to pg. 97, ll. 28)."

Independent claim 1054 recites, "1054. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising: (pg. 5, ll. 18 to pg. 6, ll. 2; pg. 25, ll. 22-34) at least one modified nucleotide or a modified nucleotide analog having the structural formula:



(pg. 2, ll. 25 to p.5, ll. 7; pg. 94, ll. 21-28) wherein BASE is a moiety comprising a pyrimidine, a pyrimidine analog, a purine, a purine analog, a deazapurine, a deazapurine analog, (pg. 90, ll. 28 to pg. 92, end; pg. 94, ll. 21 to pg. 95, end) wherein said analog can be attached to or coupled to or incorporated into DNA or RNA wherein said analog does not substantially interfere with

double helix formation or nucleic acid hybridization, (pg. 52, ll. 30 to pg. 53, ll. 4) and wherein BASE is attached to the 1' position of the furanosyl ring from the N1 position when BASE is a pyrimidine or a pyrimidine analog, from the N9 position of the furanosyl ring when BASE is a purine, a purine analog, a deazapurine or a deazapurine; (pg. 8, ll. 21-27; pg. 93, ll. 15-18) wherein x comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate; wherein y comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate; wherein z comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate; and wherein said Sig is covalently attached directly or through a chemical linkage to at least one phosphate comprising of x, y and z, or a combination thereof, (pg. 3, ll. 1-32; pg. 9, ll. 4-14; pg. 12, ll. 6-25) and wherein said Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when so attached to said phosphate (pg. 94, ll. 21-28; pg. 97, ll. 22 to pg. 98, ll. 14) or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, (pg. 95, ll. 2-11) provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2', 3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide, (pg. 53, ll. 18-27; pg. 94, ll. 21 to pg. 95, ll. 11) and wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing (pg. 82, ll. to pg. 84, ll. 10; pg. 96, ll. 22 to pg. 97, ll. 28)."

VI. Grounds of rejection to be reviewed on appeal.

The claims in the case stand rejected as follows:

- claims 955-961, 964-968, 970-976, 978-993, 996-1000, 1002-1009, 1011-1027, 1030-1034, 1036-1042, 1044-1056, 1062-1066, 1068-1075 and 1077-1087 under 35 U.S.C. § 102(b) based upon Hung et al.;
- claims 955-961, 964-968, 970-976, 978-993, 996-1000, 1002-1009, 1011-1027, 1030-1034, 1036-1042, 1044-1056, 1062-1066, 1068-1075 and 1077-1087 under 35 U.S.C. § 102(b) based upon Dunn et al.; and
- claims 956, 964-965, 971-972, 974, 978, 986, 988, 996-997, 1002-1005, 1007, 1011, 1019, 1022, 1031, 1036-1038, 1044, 1052, 1054, 1062-1063, 1068-1071, 1073, 1075, 1077, and 1085 under 35 U.S.C. § 102(a) based upon Hartman et al.

VII. Argument.

A. Legal standard.

A determination of anticipation involves two steps. First, the claims must be construed, a question of law. Next, the construed claims are compared to the prior art. *Key Pharmaceuticals v. Hercon Laboratories Corp.*, 161 F.3d 709, 48 U.S.P.Q.2d 1911, 1915 (Fed. Cir. 1998). It is well established that during patent examination, pending claims must be "given their broadest reasonable interpretation consistent with the specification." *Ex parte McCollough*, 7 U.S.P.Q.2d 1889, 1891 (BPAI 1988); *In re Hyatt*, 211 F.3d 1367, 1372, 54 U.S.P.Q.2d 1664, 1667 (Fed. Cir. 2000); *In re Sneed and Young*, 218 U.S.P.Q. 385, 388 (Fed. Cir. 1983); *see also* M.P.E.P. § 2111. The broadest reasonable interpretation of the claims must also be consistent with the interpretation that those skilled in the art would reach. *In re Morris,* 127 F.3d 1048, 1054-55, 44 U.S.P.Q.2d 1023, 1027-28 (Fed. Cir. 1997) ("PTO applies to verbiage of the proposed claims the broadest reasonable meaning of the words in their ordinary usage as they would be understood by one of ordinary skill in the art, taking into account whatever enlightenment by way of definitions or otherwise that may be afforded by the written description contained in applicant's specification."); *see also Ex parte Hoffman*, 12 U.S.P.Q.2d 1061, 1063 (BPAI 1989); M.P.E.P. § 2111.

Claim terms are presumed to have the ordinary and customary meanings attributed to them by those of ordinary skill in the art. The ordinary and customary meaning of a term may be evidenced by a variety of sources, including the intrinsic evidence and the plain meaning of the claims. *Rapoport v. Dement*, 254 F.3d 1053, 1059-60, 59 U.S.P.Q.2d 1215, 1219-20 (Fed. Cir. 2001) (Both intrinsic evidence and the plain meaning of the term "method for treatment of sleep

apneas" supported construction of the term as being limited to treatment of the underlying sleep apnea disorder itself, and not encompassing treatment of anxiety and other secondary symptoms related to sleep apnea.).

Under 35 U.S.C. § 102, the Office bears the burden of presenting at least a *prima facie* case of anticipation. *Chester v. Miller*, 906 F.2d 1574, 15 U.S.P.Q.2d 1333 (Fed. Cir. 1990); *In re Skinner*, 2 U.S.P.Q.2d 1788, 1789 (BPAI 1986). "A prior art reference anticipates a claim only if the reference discloses, either expressly or inherently, every limitation of the claim." *Rowe v. Dror*, 112 F.3d 473, 478, 42 U.S.P.Q.2d 1550, 1553 (Fed. Cir. 1997); *see also* M.P.E.P. § 2131. Absence from the reference of any claimed element negates anticipation. *Id.* (quoting *Kloster Speedsteel AB v. Crucible, Inc.*, 793 F.2d 1565, 1571, 230 U.S.P.Q. 81, 84 (Fed. Cir. 1986)). Inherent anticipation requires that the missing descriptive material is necessarily present, not merely probably or possibly present, in the prior art. *Rosco Inc. v. Mirror Lite Co.*, 304 F.3d 1373, 1380, 64 U.S.P.Q.2d 1676, 1680 (Fed. Cir. 2002), *see also Skinner*, 2 U.S.P.Q.2d at 1788-89. In addition, the prior art reference must be enabling. *Akzo N.V. v. U.S. Int'l Trade Comm'n*, 808 F.2d 1471, 1479, 1 U.S.P.Q.2d 1241, 1245 (Fed. Cir. 1986); *see also* M.P.E.P. § 2121.

B. Construction of the claims.

Appellants address the three anticipation rejections below. However, as set forth in the legal discussion above, construction of the claims is a necessary prerequisite to determining whether the prior art reads on those claims. Accordingly, Appellants begin with properly construing the claims.

The case law in this regard, as cited above, is well established. Claims during prosecution are to be given their broadest reasonable construction consistent with the

specification and with the interpretation that those skilled in the art would reach. The crux of the disagreement between the Examiner and the Appellants centers on the "broadest reasonable construction" of the claims.[1] Although any question involving what is "reasonable" is necessarily subjective, both the Board and the Federal Circuit have provided certain benchmarks by which to judge what is or is not a reasonable construction of claims.

For example, the PTO's interpretation of claim terms should not be so broad that it conflicts with the meaning given to identical terms in other patents from analogous art. *In re Cortright*, 49 U.S.P.Q.2d at (Fed. Cir. 1999). Also, where the Examiner's construction is inconsistent with the written description and the common definition of the terms as well as the examples, such a construction is not reasonable. *In re Baker Hughes Inc.*, 55 U.S.P.Q.2d 1149, 1153 (Fed. Cir. 2000). Also, where the specification indicates that a particular construction is necessary for proper functioning of the invention, it is improper for the Board to apply another construction (construing a "predetermined" gradient to be a linear gradient). *In re Roemer*, 59 U.S.P.Q.2d 1527, 1530 (Fed. Cir. 2001).

[1] The oft-stated rationale for applying the "broadest reasonable construction" standard to claims in a pending application is that, unlike issued claims in a patent, a patent applicant has the ability to amend the claims should there be a need to incorporate limitations from the specification. *See In re Bigio,* 381 F.3d 1320, 72 U.S.P.Q.2d 1209, 1211 (Fed Cir. 2004); *Bamberger v. Cheruvu,* 55 U.S.P.Q.2d 1523, 1527 (BPAI 2000). However, as this case is a Pre-GATT filing in which Appellants are precluded from filing any RCEs or continuing applications, Appellants find themselves to be more in the position of a patentee than an applicant. Indeed, the Examiner withdrew the application from issue to apply the three pieces of art currently in contention.

i. Claims' recitation of "modified nucleotide or modified nucleotide analog" does not read on unmodified polynucleotides.

The Examiner states in the November 26, 2003 Office action that Appellants' "claims read on *any DNA polymer*." [Nov. 26, 2003 Office action, at 7 (emphasis added)] According to the Examiner, every DNA polymer comprises a first and a second segment, the first segment being complementary to a target nucleic acid, and the second segment covalently attached to the first segment. In this way, the second nucleic acid segment constitutes a Sig as it is non-polypeptide, non-radioactive and "detectable via its well known UV detectability of its nucleobase content." [*Id.*] Thus, according to the Examiner's construction, every polynucleotide comprises a "modified nucleotide" in the sense that, as polymerization proceeds, each additional nucleotide could be viewed as "modifying" the preceding nucleotide.

The Examiner's overly broad contention that the claims read on any DNA polymer is erroneous and unreasonable. Appellants' claims directed to a nucleotide require two things:

1. That the nucleotide be "modified;" and
2. That the modification be a "Sig" (as defined in the claim) attached to the phosphate moiety ("PM").

The Examiner's construction eviscerates the understanding of those skilled in the art as to the meaning of a "modified" nucleotide. When a nucleotide is added to a nucleotide, just as when an amino acid is added to another amino acid or a saccharide to another saccharide, the result is a polymerization, not a modification. Thus, when a nucleotide is added to another nucleotide, one skilled in the art would speak of the product not as a modified nucleotide but as a di-nucleotide. Similarly, when an amino acid is added to another amino acid, a person skilled in

the art would not refer to the product as a modified amino acid but rather a di-peptide. And when one adds a saccharide unit to another saccharide unit, a person skilled in the art does not speak of a modified saccharide but rather a di-saccharide. On the other hand, when one adds something not normally associated with a nucleotide, an amino acid or a saccharide, one speaks of a "modified" nucleotide, amino acid or saccharide.

Thus, a normal polynucleotide, *e.g.,* DNA polymer, does not comprise any "modified" nucleotides; rather, it consists entirely of *unmodified* nucleotides. It is illogical to say that each nucleotide in a polynucleotide is somehow modified by the other nucleotides. Instead, a nucleotide "modification" denotes an alteration to the structure of that particular nucleotide, *i.e.,* the attachment, removal, substitution or deletion of some atom or group of atoms. Thus, the Examiner's construction of the term "modified" as encompassing a nucleotide attached to another nucleotide defies the ordinary usage of the terminology and is not a "reasonable" interpretation.

Moreover, even if there were any ambiguity as to the meaning of the term "modified," such ambiguity is dispelled by reference to Appellants' specification. The specification employs the word "modified" when referring to the nucleotide consistent with the ordinary understanding of a person skilled in the art. The specification makes clear that it is "essential" that the oligo- or polynucleotide of the invention *not* contain, as the modifying compound, a nucleotide modified with another nucleotide:

> Several *essential* criteria must be satisfied in order for a modified nucleotide to be generally suitable as a substitute for a radioactively-labeled form of a naturally occurring nucleotide. First, the modified compound *must* contain a substituent or probe that is unique, i.e., *not normally found associated with nucleotides*

> *or polynucleotides*... All of these criteria are satisfied by the modified nucleotides described herein. [pg. 7, ll. 1-6; pg. 8, ll. 18-19 (emphasis added)]

The specification could not be any clearer on this point—the "modified" nucleotide is suitable for the invention if it includes a substituent that is "unique." The attachment of a nucleotide to another nucleotide cannot be viewed as providing a unique substituent not normally found associated with nucleotides.

As if this clear statement of the meaning of "modified" were not sufficient to repudiate the Examiner's construction of the term, the definition of Sig in the specification necessarily precludes its being defined as a nucleotide or polynucleotide. The Examiner's construction of Sig contravenes the teachings of Appellants' specification and the evidence of record. The specification states, "As indicated *in accordance with the practices of this invention*, the Sig component could comprise any chemical moiety which is attachable either directly or through a chemical linkage or linker arm to *the nucleotide*, such as to the... P component thereof." [pg. 97, ll. 22-28 (emphasis added)] This passage contains important limiting language narrowing the scope of what "Sig" can include. It plainly omits from the definition of the Sig component all practices not "in accordance with the invention." One such practice readily recognized by a person of skill in the art as not in accordance with the invention is wherein the Sig component is itself a nucleotide or polynucleotide.[2]

[2] Appellants submitted along with their September 3, 2004 Amendment a declaration from Alex A. Waldrop, III, Ph.D, averring that, as one having ordinary skill in the art, he understood the specification as specifically, albeit implicitly, excluding "nucleotide" from the definition of Sig. It is unclear, however, whether Dr. Waldrop's declaration was ever entered into the record. Although the November 29, 2004 advisory action does not expressly indicate that the declaration was entered, the Examiner spent nearly two pages of the advisory action addressing the assertions made by Dr. Waldrop, eventually concluding that the declaration was found to be non-

The passage also makes clear that the "Sig component" of the nucleotide is distinct from "the nucleotide" itself. Indeed, if the Sig component were itself a nucleotide, any distinction between "the nucleotide component" and "the Sig component" of the resulting modified nucleotide would be rendered meaningless. This is corroborated by other passages from the specification, which is replete with evidence that "Sig-containing nucleotide" refers to a single nucleotide comprising a detectable label, and not a polynucleotide. (*See* Chart 1 at the conclusion of this section). For instance, the specification states:

> The chemical moiety Sig so attached to the nucleotide P-S-B [PM-SM-BASE] is capable of rendering or making *the resulting nucleotide*, now comprising P-S-B [PM-SM-BASE] with the Sig moiety being attached to one or more of the other moieties, self-detecting or signalling itself or capable of making its presence known per se, *when incorporated into a polynucleotide*... [pg. 96, ll. 12-19]

This passage refers to the Sig-containing nucleotide as "the resulting nucleotide." If the Sig component itself were a nucleotide or polynucleotide, this passage would make no sense. There would be no "resulting nucleotide"; rather, there would be a "resulting *poly*nucleotide" or "resulting *di*nucleotide." Furthermore, the passage distinguishes between the "resulting nucleotide" (singular) and the "polynucleotide" (plural) into which the resulting nucleotide is incorporated. To be sure, the specification repeatedly distinguishes between the Sig-containing nucleotide of the invention and polynucleotides which incorporate the Sig-containing nucleotide.

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persuasive. Presuming that the Examiner would not have addressed the declaration on its merits had he not entered it in the record, and presuming that he would have indicated non-entry of the declaration had it not been entered, Appellants accordingly treat the declaration as of record and submit it in the Evidence Appendix for the Board's convenience. [*See* Exhibit 1 of the Evidence appendix.]

(*See* Chart 2 at the conclusion of this section). The specification never suggests, explicitly or implicitly, that the Sig-containing nucleotide could be an unmodified polynucleotide.

Indeed, the specification enumerates several examples of the sort of moieties the Sig component could comprise, including an enzyme, a fluorescing component, an electron dense component, a hapten, a chemiluminescent component, etc.[3] [pg. 96, ll. 30 to pg. 31, ll. 20] Conspicuously absent from the list of possible Sig components, however, are nucleotides and nucleic acids, which were well known in the art at the time of filing. A person of ordinary skill in the art would not have viewed this omission as accidental, but as intentional for all the reasons discussed above.

Accordingly, contrary to the Examiner's contention, the claims do not read on "any DNA polymer," or on "any RNA polymer."

The following charts identify relevant portions of the specification which support Appellants' claim construction position. Chart 1 includes statements from the specification denoting that "Sig-containing nucleotide" is a single nucleotide comprising a detectable label, and not a polynucleotide. Chart 2 includes statements from the specification denoting that Sig-containing nucleotides are distinct from polynucleotides that incorporate Sig-containing nucleotides.

[3] Appellants do not intend to relinquish any equivalents unforeseeable at this time or which bear only a tangential or peripheral relation to the reason for the present amendment, which is merely to clarify that the Sig component cannot be a nucleotide.

Chart 1

Description	Location
"The Sig chemical moiety is covalently attached to the phosphoric acid P moiety via the [phosphate] chemical linkage... said Sig, when attached to said P moiety being capable of signalling itself or making itself self-detecting or its presence known and desirably *the nucleotide* is capable of being incorporated into a double-stranded polynucleotide..."	pg. 95, ll. 2-10
"The chemical moiety Sig so attached to the nucleotide P-S-B is capable of rendering or making the *resulting nucleotide*, now comprising P-S-B with the Sig moiety being attached to one or more of the other moieties, self-detecting or signalling itself or capable of making its presence known per se, when incorporated into a polynucleotide..."	pg. 96, ll. 12-19
"The Sig moiety desirably should not interfere with the capability of *the nucleotide* to form a double-stranded polynucleotide containing the *special Sig-containing nucleotide* in accordance with this invention and, when so incorporated therein, the *Sig-containing nucleotide* is capable of detection, localization or observation."	pg. 96, ll. 22-28
"As indicated, such probes may contain one or more of the *special Sig-containing nucleotides* in accordance with this invention, preferably at least about *one special nucleotide* per 5-10 of the nucleotides in the probe."	pg. 99, ll. 6-10

Chart 2

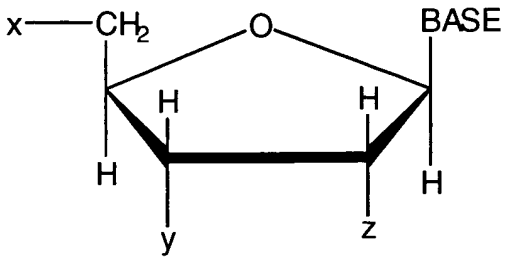
Description	Location
"The special nucleotides of this invention *and polynucleotides including such nucleotides*, either single-stranded or double-stranded polynucleotides, DNA and/or RNA, comprising the components, phosphoric acid moiety P, the sugar or monosaccharide moiety S, the base moiety B, a purine or a pyrimidine, and the signalling or self-detecting moiety, Sig, covalently attached to either the P, S or B moieties, as indicated hereinabove, have many uses and utilities."	pg. 103, ll. 10-18
"For example, the nucleotides of this invention *and polynucleotides containing the nucleotides of this invention...*"	pg. 103, ll. 18-20

"The advantage of polynucleotides, such as double-stranded *polynucleotides incorporating one or more nucleotides in accordance with this invention* is..."	pg. 103, ll. 33-35
"For example, nucleotides in accordance with this invention containing the above-described components P, S, B and Sig, are suitably prepared so that the nucleotides *and polynucleotides prepared therefrom* are more resistant to nucleases."	pg. 104, ll. 4-8
"Similarly, such nucleotides *and polynucleotides containing the same* and suitably prepared which are more capable of contacting, stimulating and penetrating cellular surfaces or membranes..."	pg. 104, ll. 8-12
"Such nucleotides *and polynucleotides incorporating the same*, both single-stranded and double-stranded nucleotides..."	pg. 104, ll. 22-24
"Increased resistance to nucleases is also achievable as well as alterations or favorable changes in the hydrophobic properties or electrical or charge properties of the nucleotides *and polynucleotides containing the same.*"	pg. 104, ll. 28-32
"The special nucleotides of this invention, *including polynucleotides containing these nucleotides...*"	pg. 105, ll. 14-15
"The moiety Sig attached to the special nucleotides of this invention containing the other moieties or components P, S, B..."	pg. 105, ll. 22-24
"The administration of the nucleotides *and/or polynucleotides containing the nucleotides* to the body ..."	pg. 106, ll. 1-2
"...[T]he special P, S, B and Sig-containing nucleotides of this invention, *including polynucleotides containing such nucleotides*, are useful as immune-stimulating agents and adjuvants therefor."	pg. 106, ll. 15-18
"Of special interest in the practices of this invention improved *polynucleotides incorporating the special nucleotides of this invention* are provided as inducers or stimulating agents for the production of interferon."	pg. 106, ll. 24-27

ii. Claims' recitation that Sig be covalently attached to the phosphate moiety "directly or through a chemical linkage" does not read on modified nucleotides in which Sig is attached directly to the base moiety.

The Examiner suggests in the November 26, 2003 Office action that Appellants' claims read on modified nucleotides to which a label is attached to the base moiety of the nucleotide. In rejecting Appellants' claims over Hartman et al., the Examiner argues that the "Sig label [of Hartman et al.] is a yellow chromogenic label which modifies the terminal poly(A) nucleotide as required in the instant claims thus similarly anticipating the instant claims..." [Nov. 26, 2003 Office action, at 6-7] Presumably, the Examiner believes that Appellants' claims, with their recitation that Sig be "covalently attached to PM directly or through *a chemical linkage*," permit the sugar and base moieties of the modified nucleotide itself to constitute the "chemical linkage" by which the Sig is attached to the phosphate moiety. This interpretation of the claims is at odds with both the plain meaning of the claims and the specification.

Independent claims 956 and 1022 are directed to modified nucleotides or nucleotide analogs having the formula *Sig—PM*—SM—BASE. The recited formula in these claims explicitly calls for the Sig moiety to be covalently attached to the *phosphate moiety*, not the base moiety of the nucleotide. Indeed, the recited formula designates that the Sig moiety is physically separated from the base moiety by both the phosphate moiety and the sugar moiety. Similarly, independent claims 988 and 1054 are directed to modified nucleotides or modified nucleotide analogs having the formula:



wherein Sig is covalently attached directly or through a chemical linkage to at least one *phosphate* comprising *x, y, z,* or a combination thereof. These claims also explicitly require Sig to be attached to a phosphate moiety comprising x, y or z, each of which is physically separated from the base moiety by the sugar moiety.

Accordingly, each of the independent claims embraces modified nucleotides in which Sig is covalently attached directly or indirectly to a phosphate moiety, the phosphate moiety is attached directly to the sugar moiety, and the sugar moiety is attached directly to the base moiety, *in that order.* Specifically excluded from the claims' scope are modified nucleotides in which Sig is attached directly to the base moiety. It would be unreasonable, based upon the plain meaning of the claims (*e.g.,* Sig—PM—SM—BM), to interpret the claims as reading on a Sig attached to the phosphate via the base (*e.g.,* PM—SM—BM—Sig).

Furthermore, the specification discloses embodiments of modified nucleotides, including wherein the Sig moiety is directly or indirectly attached to (i) the phosphate moiety, (ii) the sugar moiety or (iii) the base moiety. However, in the present application, Appellants chose to pursue only those claims directed to that particular embodiment in which Sig is attached to the *phosphate moiety*, rather than that embodiment in which the Sig is directly attached to the base moiety. Accordingly, Appellants' pending claims ought not be construed as encompassing embodiments in which the Sig is directly attached to the base moiety.

C. Prior art rejections

i. Rejection of claims 956-961, 964-968, 970-976, 978-993, 996-1000, 1002-1009, 1011-1027, 1030-1034, 1036-1042, 1044-1056, 1062-1066, 1068-1075 and 1077-1087 under 35 U.S.C. § 102(b) based upon Hung et al.[4]

The rejection of claims 956-961, 964-968, 970-976, 978-993, 996-1000, 1002-1009, 1011-1027, 1030-1034, 1036-1042, 1044-1056, 1062-1066, 1068-1075, 1077-1087 under 35 U.S.C. § 102(b) based upon Hung et al. is untenable on several bases. First, the reference fails to disclose a "modified nucleotide or modified nucleotide analog" comprising a non-radioactive Sig moiety, as recited in Appellants' claims. Second, the Examiner has failed to meet his burden of proving that the reference teaches a Sig comprising "biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing," as recited in Appellants' claims.

Hung et al. describe a method of obtaining a complete copy of the entire length of single stranded RNA into its complementary DNA (cDNA) by reverse transcription using a binding protein. [*See* Exhibit 2 of the Evidence appendix.] The patentees isolated nucleic acid binding protein from chick embryo fibroblasts chronically transformed by Rous sarcoma virus (RSV). Nucleic acid binding activity was measured by a membrane filtration technique using ^{3}H-labeled

[4] In the November 26, 2003 non-final Office action, the Examiner rejected Appellants' claims under 35 U.S.C. § 102(b) as unpatentable over Hung et al. Appellants subsequently canceled those claims and submitted the presently pending claims. In the telephonic interview conducted on March 29, 2005, the Examiner reiterated the 102(b) rejection over Hung et al., presumably applying the same arguments advanced in the November 26, 2003 non-final Office action to Appellants' presently pending claims. Accordingly, in this Appeal Brief Appellants address the Examiner's contentions raised in the November 26, 2003 non-final Office action.

polynucleotides. To evaluate the effect of the binding protein, Hung et al. incubated RSV RNA with ^{32}P-labeled nucleotides and reverse transcriptase, ran the samples on an electrophoretic gel, and exposed the gel to autoradiography to visualize the bands of ^{32}P-labeled RNA. The results indicated that reverse transcriptase from RSV was able to synthesize a complete or nearly complete DNA copy from the purified viral RNA in the presence of the binding protein isolated from the chick cells.

The Examiner contends in the November 26, 2003 Office action that Appellants' "claims read on *any DNA polymer*" or "on any other RNA polymer." [Nov. 26, 2003 Office action, at 7 (emphasis added)] According to the Examiner, because Hung et al. disclose "both RNA and DNA, as cDNA, polymers in the abstract and throughout its disclosure as part of the synthesis disclosure therein," the reference "anticipates both the instant RNA and DNA type claims listed above." [*Id.*] As discussed above, the Examiner's construction of the claims is unreasonable. A normal polynucleotide consists entirely of *unmodified* nucleotides. It is unreasonable for the Examiner to construe a polynucleotide as simply a modified nucleotide. A person skilled in the art would appreciate that the term "modified nucleotide" denotes an alteration to the structure of that particular nucleotide, *i.e.,* the attachment, removal, substitution or deletion of some atom or group of atoms, not simply the attachment of more nucleotides.

Contrary to the Examiner's contention, the claims do not read on "any DNA polymer" or "any RNA polymer". As such, the non-radioactive polynucleotides taught by Hung et al. fail to anticipate the claims, which require one or more "modified" nucleotides. The only "modified nucleotides" disclosed in Hung et al. are ^{3}H- and ^{32}P-labeled nucleotides, which are

radioactively-labeled nucleotides and thus fall outside the scope of the claims, which recite that Sig is "non-radioactive".[5]

The Examiner has failed to make out a *prima facie* case of anticipation. In particular, the Examiner has not met his burden of proving that Hung et al. teaches a Sig comprising "biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing," as recited in Appellants' claims. According to the Examiner, the Sig disclosed in Hung et al. is a polynucleotide, and as such it is "detectable via its well known *UV detectability* of its nucleobase content." [Nov. 26, 2003 Office action, at 7 (emphasis added)] The Examiner's conclusion, however, is nothing more than a naked allegation without evidence that the polynucleotides disclosed in Hung et al. are in fact UV detectable.

Accordingly, the Examiner has failed to meet his burden of proving that Hung et al. teaches a Sig comprising "biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing," as recited in Appellants' claims.

[5] It is worth pointing out that the Examiner has not rejected any claims over the radioactively-labeled polynucleotide disclosed in Hung et al.

ii. Rejection of claims 956-961, 964-968, 970-976, 978-993, 996-1000, 1002-1009, 1011-1027, 1030-1034, 1036-1042, 1044-1056, 1062-1066, 1068-1075 and 1077-1087 under 35 U.S.C. § 102(b) based upon Dunn et al.[6]

The rejection of claims 956-961, 964-968, 970-976, 978-993, 996-1000, 1002-1009, 1011-1027, 1030-1034, 1036-1042, 1044-1056, 1062-1066, 1068-1075, 1077-1087 under 35 U.S.C. § 102(b) based upon Dunn et al. is likewise untenable. Dunn et al. teaches a two-step hybridization procedure. [*See* Exhibit 3 of the Evidence appendix.] In the first step, RNA extracted from cells infected with an adenovirus-SV40 hybrid is hybridized to adenovirus type 2 (Ad2) DNA immobilized on nitrocellulose filters. RNA molecules containing both Ad2 and SV40 sequences form duplexes through complementary Ad2 sequences, leaving their SV40 sequences as protruding tails. In the second step of the procedure, the tailed Ad2-SV40 hybrids are annealed to ^{32}P-labeled SV40 DNA, resulting in labeled tails which permit the autoradiographic identification of the sequences of Ad2 homologous to the RNA.

The Examiner states in the November 26, 2003 Office action that claim 627[7] is unpatentable over Dunn et al. because "Dunn et al. at page 24 in Figure 1 depicts an adenovirus

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[6] In the November 26, 2003 non-final Office action, the Examiner rejected Appellants' claims under 35 U.S.C. § 102(b) as unpatentable over Dunn et al. Appellants subsequently canceled those claims and submitted the presently pending claims. In the telephonic interview conducted on March 29, 2005, the Examiner reiterated the 102(b) rejection over Dunn et al., presumably applying the same arguments advanced in the November 26, 2003 non-final Office action to Appellants' presently pending claims. Accordingly, in this Appeal brief Appellants address the Examiner's contentions raised in the November 26, 2003 non-final Office action.

[7] Claim 627, now canceled, is substantially similar to pending independent claims 956, 988, 1022 and 1054 for purposes of this rejection. In particular, canceled claim 627, like the pending claims, is drawn to an oligo- or polynucleotide comprising a modified nucleotide wherein Sig is covalently attached to a phosphate moiety directly or through a chemical linkage, wherein Sig comprises a non-polypeptide, non-radioactive label moiety, and wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing

RNA/SV40 RNA conjugate wherein the SV40 RNA is non-polypeptide and non-radioactive and attached via a phosphate to the terminal adenovirus RNA oligonucleotide which is complementary to the immobilized Ad2 DNA." [Nov. 26, 2003 Office action, at 6] The Examiner adds that "the Sig SV40 RNA is chromogenic as being well known to be detectable via UV light." [Nov. 26, 2003 Office action, at 6] In other words, according to the Examiner, the SV40 RNA taught in Dunn et al. is inherently "chromogenic," and as such it reads on the instantly claimed "Sig" detectable label.

The Examiner fails to set forth a *prima facie* case of anticipation of these claims as Dunn et al. does not disclose each and every element of the claimed invention. In particular, Dunn et al. does not disclose an oligo- or polynucleotide comprising a "modified nucleotide", as recited in the claims. The Examiner's contention of unpatentability rests on the mistaken notion that the adenovirus RNA-SV40 RNA disclosed in Dunn et al. somehow constitutes a modified nucleotide. It does not. Rather, Dunn et al.'s RNA is a *poly*nucleotide. As explained more fully above, a person skilled in the art would understand that a nucleotide "modification" denotes an alteration to the structure of that particular nucleotide; it does not denote the addition of more nucleotides. Indeed, the only *modified* nucleotides disclosed in Dunn et al. are ^{32}P-labeled SV40 DNA, which are specifically excluded from the claims, which recite that Sig is "non-radioactive".[8]

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component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing.

[8] The Examiner has not rejected any of Applicant's claims over the radioactively-labeled polynucleotides disclosed in Dunn et al.

Moreover, the Examiner has failed to meet his burden of proving that the SV40 RNA disclosed by Dunn et al., which the Examiner contends reads on Sig, comprises "biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing," as recited in the claims. Without tendering a single supporting reference, the Examiner states in conclusory fashion that "the Sig SV40 RNA [of Dunn et al.] is chromogenic as being well known to be detectable via UV light." [Nov. 26, 2003 Office action, at 6] *See, e.g., In re Skinner*, 2 U.S.P.Q.2d 1788, 1789 (BPAI 1986) (Examiner who has reason to believe that novelty-establishing limitation may be inherent characteristic of prior art must provide some evidence or scientific reasoning to establish reasonableness of such belief before applicant can be required to demonstrate that subject matter shown to be in prior art does not possess characteristic relied upon).

Accordingly, the Examiner has not met his burden of proving that Dunn et al. teaches a "modified nucleotide or nucleotide analog" comprising a Sig "chromogenic component," as recited in Appellants' claims.

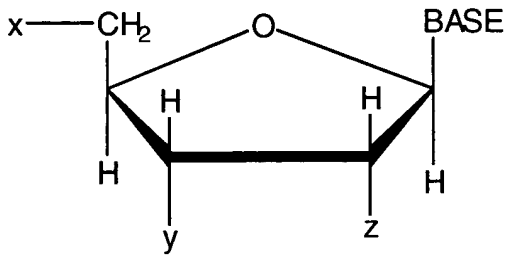
iii. Rejection of claims 956, 964-965, 971-972, 974, 978, 986, 988, 996-997, 1002-1005, 1007, 1011, 1019, 1022, 1031, 1036-1038, 1044, 1052, 1054, 1062-1063, 1068-1071, 1073, 1075, 1077, and 1085 under 35 U.S.C. § 102(a) based upon Hartman et al.[9]

The rejection of claims 956, 964-965, 971-972, 974, 978, 986, 988, 996-997, 1002-1005, 1007, 1011, 1019, 1022, 1031, 1036-1038, 1044, 1052, 1054, 1062-1063, 1068-1071, 1073, 1075, 1077, and 1085 under 35 U.S.C. § 102(a) based upon Hartman et al. is similarly without merit. Hartman et al. prepare "modified nucleotides" in which an azo group is attached to the base moiety of a nucleotide. [*See* Exhibit 4 of the Evidence appendix.] These azo-labeled nucleotides, when attached to RNA molecules complementary to a chromosomal DNA segment, permit localization of the DNA segment following *in situ* hybridization of the probe, as well as methylacrylate polymerization and marker attachment. Hartman et al. teach that these azo-nucleotides produce a characteristic peak at approximately 350 nm in the uv-absorption spectrum, resulting in a yellow-orange appearance.

The Examiner states in the November 26, 2003 Office action that the "Sig label [of Hartman et al.] is a yellow chromogenic label which modifies the terminal poly(A) nucleotide as required in the instant claims thus similarly anticipating the instant claims as the above Dunn et al. reference." [Nov. 26, 2003 Office action, at 6-7] Notwithstanding that Appellants' claims are drawn to *phosphate-labeled* nucleotides and Hartman et al. only disclose *base-labeled* nucleotides, the Examiner suggests that Appellants' claims are broad enough to encompass the

[9] In the November 26, 2003 non-final Office action, the Examiner rejected Appellants' claims under 35 U.S.C. § 102(b) as unpatentable over Hartman et al. Appellants subsequently canceled those claims and submitted the presently pending claims. In the telephonic interview conducted on March 29, 2005, the Examiner reiterated the 102(b) rejection over Hartman et al., presumably applying the same arguments advanced in the November 26, 2003 non-final Office action to

base-labeled nucleotides disclosed by Hartman et al. As explained above, this interpretation of the claim is unreasonable as it contradicts both the plain meaning of the claims and the specification. Appellants specifically chose to pursue claims directed to phosphate-labeled nucleotides. In the present application, Appellants canceled claims directed to those embodiments in which the Sig is directly attached to the base moiety. Furthermore, independent claims 956 and 1022 recite that the modified nucleotides or modified nucleotide analogs of the invention have the formula *Sig—PM*—SM—BASE. Independent claims 988 and 1054 recite that the modified nucleotides or modified nucleotide analogs have the formula



wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate comprising *x, y, z,* or a combination thereof. The formulas recited in these claims explicitly require that the Sig moiety be *physically separated* from the base moiety by both a phosphate moiety and a sugar moiety. The claims expressly prohibit Sig being attached directly to the base moiety of a nucleotide.

Based upon the plain meaning of the claim language, it would therefore be unreasonable to read the claims on Hartman et al.'s nucleotides, which comprise an azo group attached to the base moiety rather than the phosphate moiety. Unlike Appellants' nucleotides, Hartman et al.'s nucleotides require the Sig be physically separated from the phosphate by both the sugar and

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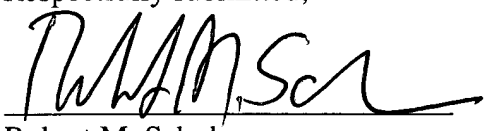
Appellants' presently pending claims. Accordingly, in this Appeal brief Appellants address the

base moieties. Accordingly, the nucleotides disclosed by Hartman et al. do not anticipate Appellants' claims.

Examiner's contentions raised in the November 26, 2003 non-final Office action.

For the foregoing reasons, reversal of the Examiner's rejections of claims 965-1087 is respectfully requested.

Respectfully submitted,



Date: April 4, 2005

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VIII. Claims appendix .

956. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide or modified nucleotide analog having the formula

Sig—PM—SM—BASE

wherein PM is a phosphate moiety, SM is a furanosyl moiety and BASE is a base moiety comprising a pyrimidine, a pyrimidine analog, a purine, a purine analog, a deazapurine or a deazapurine analog wherein said analog can be attached to or coupled to or incorporated into DNA or RNA wherein said analog does not substantially interfere with double helix formation or nucleic acid hybridization, said PM being attached to SM, said BASE being attached to SM, and said Sig being covalently attached to PM directly or through a chemical linkage, and wherein said Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polynucleotide or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, and wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing.

957. The oligo- or polynucleotide of claim 956, wherein Sig comprises at least three carbon atoms.

958. The oligo- or polynucleotide of claim 956, wherein said magnetic component

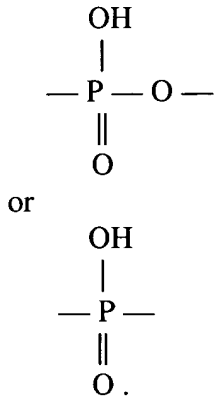
comprises magnetic oxide.

959. The oligo- or polynucleotide of claim 958, wherein said magnetic oxide comprises ferric oxide.

960. The oligo- or polynucleotide of claim 956, wherein said metal-containing component is catalytic.

961. The oligo- or polynucleotide of claim 956, wherein said fluorescent component comprises fluorescein, rhodamine or dansyl.

964. The oligo- or polynucleotide of claim 956, wherein said covalent attachment comprises



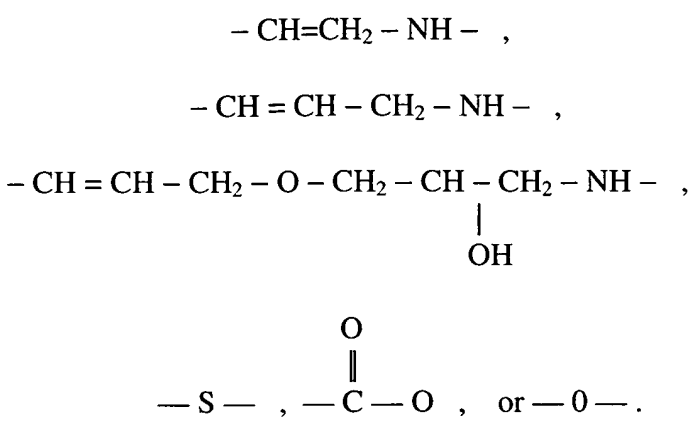
or

965. The oligo- or polynucleotide of claim 956, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

966. The oligo- or polynucleotide of claim 956, wherein said chemical linkage comprises a $-CH_2NH-$ moiety.

967. The oligo- or polynucleotide of claim 956, wherein said chemical linkage comprises an allylamine group.

968. The oligo- or polynucleotide of claim 956, wherein said chemical linkage comprises any of the moieties:



970. The oligo- or polynucleotide of claim 956, wherein said PM comprises a monophosphate, a diphosphate or a triphosphate.

971. The oligo- or polynucleotide of claim 956, wherein said Sig moiety is covalently attached to said PM through a phosphorus atom or phosphate oxygen.

972. The oligo- or polynucleotide of claim 956, wherein said Sig moiety is attached to the PM of a terminal nucleotide in said oligo- or polydeoxyribonucleotide.

973. The oligo- or polynucleotide of claim 972, wherein the furanosyl moiety of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

974. The oligo- or polynucleotide of claim 972, wherein the furanosyl moiety of said terminal nucleotide comprises an oxygen atom at the 2' position thereof.

975. The oligo- or polynucleotide of claim 973, wherein the furanosyl moiety of said terminal nucleotide comprises a hydrogen atom at the 3' position thereof.

976. The oligo- or polynucleotide of claim 974, wherein the furanosyl moiety of said

terminal nucleotide comprises an oxygen atom at the 3' position thereof.

978. The oligo- or polynucleotide of claim 956, wherein said furanosyl moiety comprises a ribose, a deoxyribose or a dideoxyribose.

979. The oligo- or polynucleotide of claim 956, wherein said pyrimidine analogs comprise thymidine analogs, uridine analogs, deoxyuridine analogs, cytidine analogs, deoxycytidine analogs or a combination of any of the foregoing.

980. The oligo- or polynucleotide of claim 979, wherein said uridine analogs comprise 5-bromo-2'-deoxyuridine-5'-phosphate.

981. The oligo- or polynucleotide of claim 979, wherein said deoxycytidine analogs comprise 5-hydroxymethyl-2'-deoxycytidylic acid.

982. The oligo- or polynucleotide of claim 956, wherein said purine analogs comprise adenosine analogs, deoxyadenosine analogs, guanosine analogs, deoxyguanosine analogs or a combination of any of the foregoing.

983. The oligo- or polynucleotide of claim 982, wherein said adenosine analogs comprise tubericidin and toyocamycin.

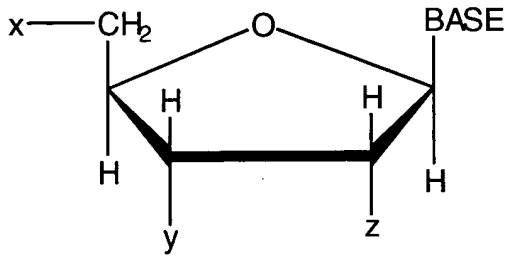
984. The oligo- or polynucleotide of claim 956, wherein said oligo- or polynucleotide comprises an oligo- or polydeoxyribonucleotide.

985. The oligo- or polynucleotide of claim 956, wherein said oligo- or polynucleotide comprises an oligo- or polydeoxyribonucleotide and further comprises at least one ribonucleotide.

986. The oligo- or polynucleotide of claim 956, wherein said oligo- or polynucleotide comprises an oligo- or polyribonucleotide.

987. The oligo- or polynucleotide of claim 956, wherein said oligo- or polynucleotide comprises an oligo- or polyribonucleotide and further comprises at least one deoxyribnucleotide.

988. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide or a modified nucleotide analog having the structural formula:



wherein BASE is a moiety comprising a pyrimidine, a pyrimidine analog, a purine, a purine analog, a deazapurine or a deazapurine analog, wherein said analog can be attached to or coupled to or incorporated into DNA or RNA, wherein said analog does not substantially interfere with double helix formation or nucleic acid hybridization, and wherein said BASE is attached to the 1' position of the furanosyl ring from the N1 position when said BASE is a pyrimidine or a pyrimidine analog, or from the N9 position when said BASE is a purine, a purine analog, a deazapurine or a deazapurine analog;

wherein x comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate;

wherein y comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate;

wherein z comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-

phosphate; and

wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate comprising x, y, z, or a combination thereof, and wherein said Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to said phosphate or when said modified nucleotide is incorporated into said oligo- or polynucleotide or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing.

989. The oligo- or polynucleotide of claim 988, wherein Sig comprises at least three carbon atoms.

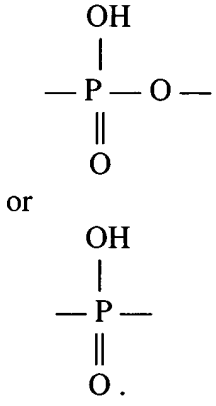
990. The oligo- or polynucleotide of claim 988, wherein said magnetic component comprises magnetic oxide.

991. The oligo- or polynucleotide of claim 990, wherein said magnetic oxide comprises ferric oxide.

992. The oligo- or polynucleotide of claim 988, wherein said metal-containing component is catalytic.

993. The oligo- or polynucleotide of claim 988, wherein said fluorescent component comprises fluorescein, rhodamine or dansyl.

996. The oligo- or polynucleotide of claim 988, wherein said covalent attachment comprises

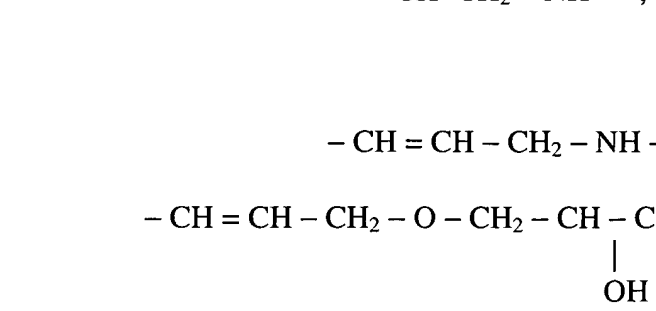


997. The oligo- or polynucleotide of claim 988, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

998. The oligo- or polynucleotide of claim 988, wherein said chemical linkage comprises a $-CH_2NH-$ moiety.

999. The oligo- or polynucleotide of claim 988, wherein said chemical linkage comprises an allylamine group.

1000. The oligo- or polynucleotide of claim 988, wherein said chemical linkage comprises any of the moieties:



1002. The oligo- or polynucleotide of claim 988, wherein said x comprises a monophosphate, a diphosphate or a triphosphate and y comprises a monophosphate.

1003. The oligo- or polynucleotide of claim 988, wherein said Sig moiety is covalently attached to said phosphate through a phosphorus atom or phosphate oxygen.

1004. The oligo- or polynucleotide of claim 988, wherein said x comprises a monophosphate.

1005. The oligo- or polynucleotide of claim 988, wherein said Sig moiety is attached to the phosphate moiety of a terminal nucleotide in said oligo- or polynucleotide.

1006. The oligo- or polynucleotide of claim 1005, wherein z of said furanosyl moiety of said terminal nucleotide comprises a hydrogen atom.

1007. The oligo- or polynucleotide of claim 1005, wherein z of said furanosyl moiety of said terminal nucleotide comprises an oxygen atom.

1008. The oligo- or polynucleotide of claim 1006, wherein y of said furanosyl moiety comprises a hydrogen atom.

1009. The oligo- or polynucleotide of claim 1007, wherein y of said furanosyl moiety comprises an oxygen atom.

1011. The oligo- or polynucleotide of claim 988, wherein said furanosyl moiety comprises a ribose, a deoxyribose or a dideoxyribose.

1012. The oligo- or polynucleotide of claim 988, wherein said pyrimidine analogs comprise thymidine analogs, uridine analogs, deoxyuridine analogs, cytidine analogs, deoxycytidine analogs or a combination of any of the foregoing.

1013. The oligo- or polynucleotide of claim 1012, wherein said uridine analogs comprise 5-bromo-2'-deoxyuridine-5'-phosphate.

1014. The oligo- or polynucleotide of claim 1012, wherein said deoxycytidine analogs comprise 5-hydroxymethyl-2'-deoxycytidylic acid.

1015. The oligo- or polynucleotide of claim 988, wherein said purine analogs comprise adenosine analogs, deoxyadenosine analogs, guanosine analogs, deoxyguanosine analogs, or a combination of any of the foregoing.

1016. The oligo- or polynucleotide of claim 1015, wherein said adenosine analogs comprise tubericidin or toyocamycin.

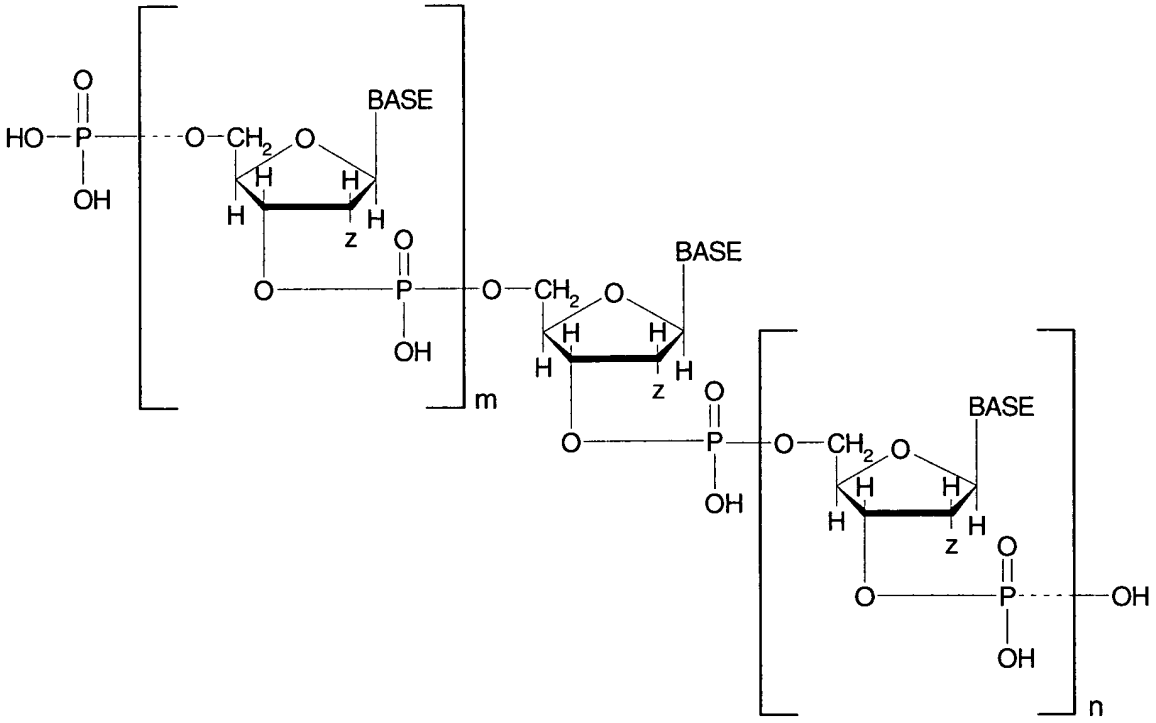
1017. The oligo- or polynucleotide of claim 988, wherein said oligo- or polynucleotide comprises an oligo- or polydeoxyribonucleotide.

1018. The oligo- or polynucleotide of claim 988, wherein said oligo- or polynucleotide comprises an oligo- or polydeoxyribonucleotide and further comprises at least one ribonucleotide.

1019. The oligo- or polynucleotide of claim 988, wherein said oligo- or polynucleotide comprises an oligo- or polyribonucleotide.

1020. The oligo- or polynucleotide of claim 988, wherein said oligo- or polynucleotide comprises an oligo- or polyribonucleotide and further comprises at least one deoxyribonucleotide.

1021. The oligo- or polynucleotide of claim 988, having the structural formula:



wherein m and n represent integers from 0 up to about 100,000, and wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula.

1022. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide or a modified nucleotide analog having the formula

Sig – PM – SM – BASE

wherein PM is a phosphate moiety, SM is a furanosyl moiety and BASE is a base moiety comprising a pyrimidine, a pyrimidine analog, a purine, a purine analog, a deazapurine or a deazapurine analog, wherein said analog can be attached to or coupled to or incorporated into DNA or RNA, wherein said analog does not substantially interfere with double helix formation or nucleic acid hybridization, said PM is attached to SM, said

BASE is attached to SM, said Sig is covalently attached to PM directly or via a chemical linkage, and wherein said Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide, and wherein said Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing.

1023. The oligo- or polynucleotide of claim 1022, wherein Sig comprises at least three carbon atoms.

1024. The oligo- or polynucleotide of claim 1022, wherein said magnetic component comprises magnetic oxide.

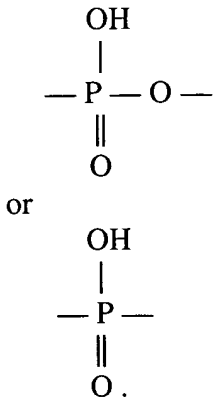
1025. The oligo- or polynucleotide of claim 1024, wherein said magnetic oxide comprises ferric oxide.

1026. The oligo- or polynucleotide of claim 1022, wherein said metal-containing component is catalytic.

1027. The oligo- or polynucleotide of claim 1022, wherein said fluorescent component

comprises fluorescein, rhodamine or dansyl.

1030. The oligo- or polynucleotide of claim 1022, wherein said covalent attachment comprises



or

1031. The oligo- or polynucleotide of claim 1022, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

1032. The oligo- or polynucleotide of claim 1022, wherein said chemical linkage comprises a -CH_2NH- moiety.

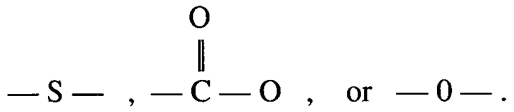
1033. The oligo- or polynucleotide of claim 1022, wherein said chemical linkage comprises an allylamine group.

1034. The oligo- or polynucleotide of claim 1022, wherein said chemical linkage comprises any of the moieties:

$$-CH{=}CH_2-NH- \quad ,$$

$$-CH=CH-CH_2-NH- \quad ,$$

$$-CH=CH-CH_2-O-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-NH- \quad ,$$



1036. The oligo- or polynucleotide of claim 1022, wherein said PM comprises a monophosphate, a diphosphate or a triphosphate.

1037. The oligo- or polynucleotide of claim 1022, wherein said Sig moiety is covalently attached to said PM through a phosphorus atom or phosphate oxygen.

1038. The oligo- or polynucleotide of claim 1022, wherein said Sig moiety is attached to the PM of a terminal nucleotide in said oligo- or polynucleotide.

1039. The oligo- or polynucleotide of claim 1038, wherein the furanosyl moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.

1040. The oligo- or polynucleotide of claim 1038, wherein the furanosyl moiety of said terminal nucleotide has an oxygen atom at the 2' position thereof.

1041. The oligo- or polynucleotide of claim 1039, wherein the furanosyl moiety of said terminal nucleotide has a hydrogen atom at the 3' position thereof.

1042. The oligo- or polynucleotide of claim 1040, wherein the furanosyl moiety of said terminal nucleotide has an oxygen atom at the 3' position thereof.

1044. The oligo- or polynucleotide of claim 1022, wherein said furanosyl moiety comprises a ribose, a deoxyribose or a dideoxyribose.

1045. The oligo- or polynucleotide of claim 1022, wherein said pyrimidine analogs comprise thymidine analogs, uridine analogs, deoxyuridine analogs, cytidine analogs, deoxycytidine analogs or a combination of any of the foregoing.

1046. The oligo- or polynucleotide of claim 1045, wherein said uridine analogs comprise 5-bromo-2'-deoxyuridine-5'-phosphate.

1047. The oligo- or polynucleotide of claim 1045, wherein said deoxycytidine analogs comprise 5-hydroxymethyl-2'-deoxycytidylic acid.

1048. The oligo- or polynucleotide of claim 1022, wherein said purine analogs comprise adenosine analogs, deoxyadenosine analogs, guanosine analogs, deoxyguanosine analogs or a combination of any of the foregoing.

1049. The oligo- or polynucleotide of claim 1048, wherein said adenosine analogs comprise tubericidin and toyocamycin.

1050. The oligo- or polynucleotide of claim 1022, wherein said oligo- or polynucleotide comprises an oligo- or polydeoxyribonucleotide.

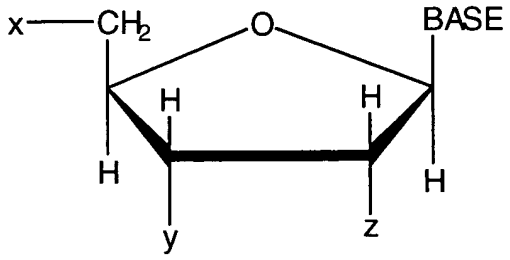
1051. The oligo- or polynucleotide of claim 1022, wherein said oligo- or polynucleotide comprises an oligo- or polydeoxyribonucleotide and further comprises at least one ribonucleotide.

1052. The oligo- or polynucleotide of claim 1022, wherein said oligo- or polynucleotide comprises an oligo- or polyribonucleotide.

1053. The oligo- or polynucleotide of claim 1022, wherein said oligo- or polynucleotide comprises an oligo- or polyribonucleotide and further comprises at least one deoxyribnucleotide.

1054. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide or a modified nucleotide analog having the structural formula:

Enz-5(D6)(C2)



wherein BASE is a moiety comprising a pyrimidine, a pyrimidine analog, a purine, a purine analog, a deazapurine, a deazapurine analog, wherein said analog can be attached to or coupled to or incorporated into DNA or RNA wherein said analog does not substantially interfere with double helix formation or nucleic acid hybridization, and wherein BASE is attached to the 1' position of the furanosyl ring from the N1 position when BASE is a pyrimidine or a pyrimidine analog, from the N9 position of the furanosyl ring when BASE is a purine, a purine analog, a deazapurine or a deazapurine;

wherein x comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate;

wherein y comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate;

wherein z comprises H— , HO— , a mono-phosphate, a di-phosphate or a tri-phosphate; and

wherein said Sig is covalently attached directly or through a chemical linkage to at least one phosphate comprising of x, y and z, or a combination thereof, and wherein said Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when so attached to said phosphate or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is

hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide, and wherein Sig comprises biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing.

1055. The oligo- or polynucleotide of claim 1054, wherein Sig comprises at least three carbon atoms.

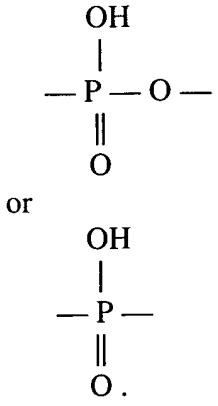
1056. The oligo- or polynucleotide of claim 1054, wherein said magnetic component comprises magnetic oxide.

1057. The oligo- or polynucleotide of claim 1056, wherein said magnetic oxide comprises ferric oxide.

1058. The oligo- or polynucleotide of claim 1054, wherein said metal-containing component is catalytic.

1059. The oligo- or polynucleotide of claim 1054, wherein said fluorescent component comprises fluorescein, rhodamine or dansyl.

1062. The oligo- or polynucleotide of claim 1054, wherein said covalent attachment comprises

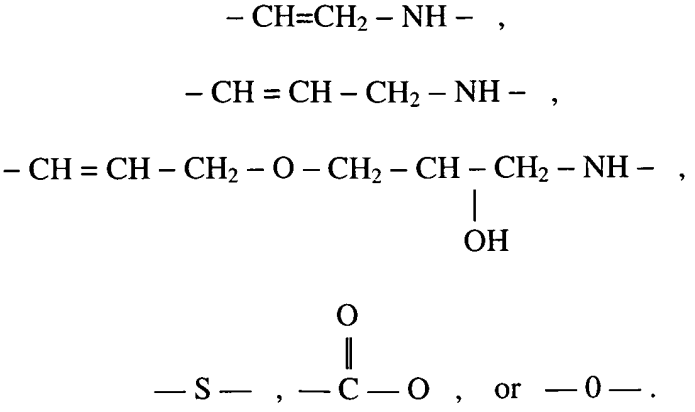


1063. The oligo- or polynucleotide of claim 1054, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

1064. The oligo- or polynucleotide of claim 1054, wherein said chemical linkage comprises a -CH_2NH- moiety.

1065. The oligo- or polynucleotide of claim 1054, wherein said chemical linkage comprises an allylamine group.

1066. The oligo- or polynucleotide of claim 1054, wherein said chemical linkage comprises any of the moieties:



1068. The oligo- or polynucleotide of claim 1054, wherein said x comprises a monophosphate, a diphosphate or a triphosphate and y comprises a monophosphate.

1069. The oligo- or polynucleotide of claim 1054, wherein said Sig moiety is covalently attached to said phosphate through a phosphorus atom or phosphate oxygen.

1070. The oligo- or polynucleotide of claim 1054, wherein said x comprises a monophosphate.

1071. The oligo- or polynucleotide of claim 1054, wherein said Sig moiety is attached to the phosphate moiety of a terminal nucleotide in said oligo- or polynucleotide.

1072. The oligo- or polynucleotide of claim 1071, wherein z of said furanosyl moiety of said terminal nucleotide comprises a hydrogen atom.

1073. The oligo- or polynucleotide of claim 1071, wherein z of said furanosyl moiety of said terminal nucleotide comprises an oxygen atom.

1074. The oligo- or polynucleotide of claim 1072, wherein y of said furanosyl moiety comprises a hydrogen atom.

1075. The oligo- or polynucleotide of claim 1073, wherein y of said furanosyl moiety comprises an oxygen atom.

1077. The oligo- or polynucleotide of claim 1054, wherein said furanosyl moiety comprises a ribose, a deoxyribose or a dideoxyribose.

1078. The oligo- or polynucleotide of claim 1054, wherein said pyrimidine analogs comprise thymidine analogs, uridine analogs, deoxyuridine analogs, cytidine analogs, deoxycytidine analogs or a combination of any of the foregoing.

1079. The oligo- or polynucleotide of claim 1078, wherein said uridine analogs comprise 5-bromo-2'-deoxyuridine-5'-phosphate.

1080. The oligo- or polynucleotide of claim 1078, wherein said deoxycytidine analogs comprise 5-hydroxymethyl-2'-deoxycytidylic acid.

1081. The oligo- or polynucleotide of claim 1054, wherein said purine analogs comprise adenosine analogs, deoxyadenosine analogs, guanosine analogs, deoxyguanosine analogs, or a combination of any of the foregoing.

1082. The oligo- or polynucleotide of claim 1081, wherein said adenosine analogs comprise tubericidin or toyocamycin.

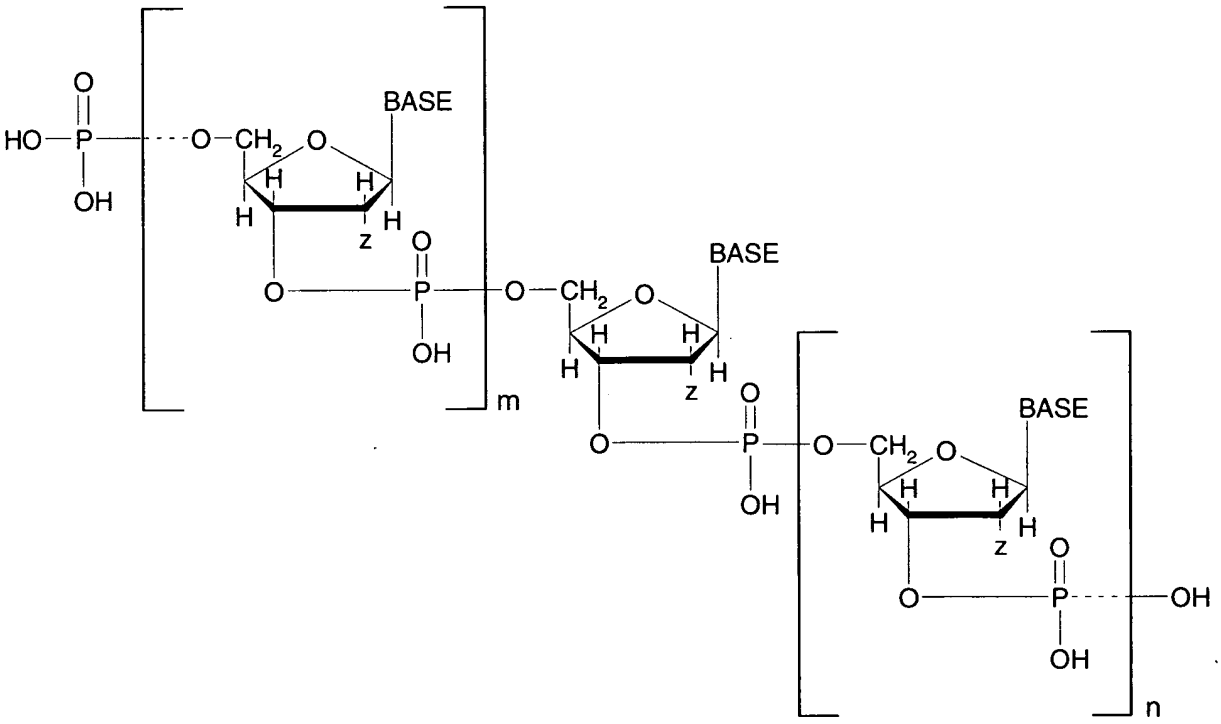
1083. The oligo- or polynucleotide of claim 1054, wherein said oligo- or polynucleotide comprises an oligo- or polydeoxyribonucleotide.

1084. The oligo- or polynucleotide of claim 1083, wherein said oligo- or polynucleotide comprises an oligo- or polydeoxyribonucleotide and further comprises at least one ribonucleotide.

1085. The oligo- or polynucleotide of claim 1054, wherein said oligo- or polynucleotide comprises an oligo- or polyribonucleotide.

1086. The oligo- or polynucleotide of claim 1054, wherein said oligo- or polynucleotide comprises an oligo- or polyribonucleotide and further comprises at least one deoxyribnucleotide.

1087. The oligo- or polynucleotide of claim 1054, having the structural formula:



wherein m and n represent integers from 0 up to about 100,000, and wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula.

IX. Evidence appendix.

Exhibit 1 Supplemental Declaration of Dr. Alex A. Waldrop, III, submitted September 3, 2004, and entered November 29, 2004.[10]

Exhibit 2 Hung et al., U.S. Patent No. 4,224,408, entered November 26, 2003.

Exhibit 3 Dunn et al. *Cell*, Vol. 12, 23-36 (1977), entered November 26, 2003.

Exhibit 4 Hartman et al., *Biopolymers*, Vol. 20, 2635-2648 (1981), entered November 26, 2003.

[10] *See* footnote 2, *supra*.

X. Related proceedings appendix.

None.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Engelhardt, *et al.*	
Serial No.:	08/479,997	Group Art Unit: 1631
Filed:	June 7, 1995	Ex'r: Ardin H. Marschel, Ph.D.
For:	OLIGO- OR POLYNUCLEOTIDES COMPRISING PHOSPHATE MOIETY LABELED NUCLEOTIDES (As Previously Amended)	

South Portland, Maine 04106

Commissioner for Patents
Washington, D.C. 20231

SUPPLEMENTAL DECLARATION OF DR. ALEX A. WALDROP, III

I, Alex A. Waldrop, III, hereby declare as follows:

1. I am the same Dr. Alexander A. Waldrop, III who submitted a Declaration in the above-identified application in June 2002. My professional background, education, training and experience are as described in my *curriculum vitae* (cv) attached as Exhibit 1 to my June 2002 Declaration. A recent cv is attached, however, as Exhibit A to this Supplemental Declaration.

2. Enzo Life Sciences, Inc. has requested that I review as its scientific consultant significant portions of the most recent prosecution history of United States Patent Application Serial No. 08/479,997, filed on June 7, 1995 ("the '997

application") in the name of Dean L. Engelhardt, *et al.* as inventors. The title of the Engelhardt application is "Oligo- or Polynucleotides Comprising Phosphate Moiety Labeled Nucleotides." Included for this particular review were the following documents:

- two Office Actions dated July 14, 2004 and November 26, 2003;
- four documents cited in the November 26, 2003 Office Action:
    - Lehninger, Biochemistry, Worth Publishers, Inc., New York, NY, 1970, pages 638-639;
    - Hartman et al., "Methacrylate Polymerization by AzoRNA: Potential Usefulness for Chromosomal Localization of Genes," Biopolymers 20:2635-2648 (1981);
    - Dunn et al., "A Novel Method to Map Transcripts: Evidence for Homology between an Adenovirus mRNA and Discrete Multiple Regions of the Viral Genome," Cell 12:23-36 (1977); and
    - Hung et al., U.S. Patent No. 4,224,408;
- Applicants' April 23, 2004 Amendment Under 37 C.F.R. §1.115 (In Response To the November 26, 2003 Office Action);[1]
- A set of claims that are being submitted in response to the July 14, 2004 Office Action;[2,3]

[1] I understand that the previously pending claims, 826-1227, were submitted in Applicants' April 23, 2004 Amendment.

[2] Copy attached as Exhibit B.

[3] The set includes claims 826, 828-832, 835-847, 849-856, 858-862, 865-878, 880-888, 890-894, 897-909, 911-921, 923-927, 930-943, 945-956, 958-961, 964-976, 978-988, 990-993, 996-1009, 1011-1022, 1024-1027, 1030-1042, 1044-1054, 1056-1059, 1062-1075, 1077-1088, 1090-1094, 1097-1112, 1114-1121, 1123-1127, 1130-1146, 1148-1156, 1158-1162, 1165-1177, 1179-1191, 1193-1197, 1200-1213 and 1215-1227. Twelve claims (826, 856, 888, 921, 956, 988, 1022, 1054, 1088, 1121, 1156 and 1191) are independent and each of these has been amended.

a fluorescent component, a chemiluminescent component, a chromogenic component, or a combination of any of the foregoing.

4. I have read the July 14, 2004 Office Action in which the Patent Examiner rejected all of the claims, 826-1227, as failing to comply with the written description requirement because of a "'non-nucleotidyl' limitation directed to the Sig moiety species."[5]

5. As Enzo's scientific consultant, I am making this Supplemental Declaration in support of the subject matter claimed in the '997 application, and in particular, to the definition recited in the claims that the non-radioactive Sig component is "non-nucleotidyl." I have been informed that my Supplemental Declaration will be submitted to the U.S. Patent Office as part of a response to the July 14, 2004 Office Action.

6. As set forth in my previously submitted cv, I am a chemist with substantial experience and background in nucleic acid chemistry. My knowledge, background, training and experience in nucleic acid chemistry encompasses nucleic acid modifications, including labeling nucleic acids for use in hybridization and detection

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[5] The full text of the Examiner's comments is set forth on page 2 in the July 14, 2004 Office Action and provides:

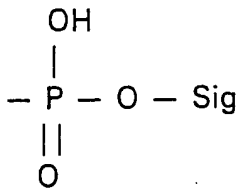
> **The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.**
>
> **NEW MATTER has been newly added to the claims due to the added "non-nucleotidyl" limitation directed to Sig moiety species. Applicants have not pointed to written basis as filed for this limitation nor has written basis as filed been found via consideration of the entirety of the instant disclosure as filed. It is acknowledged that numerous types of Sig moieties have been exemplified as filed, however, a clear delineation of such moieties to support the "non-nucleotydl" limitation has not been found. All independent claims contain this NEW MATTER limitation. Claims which depend from independent claims either directly or indirectly also contain this NEW MATTER due to their dependence. This rejection is necessitated by amendment.**

nucleotidyl Sig component. For reasons which are given below, I believe that the '997 specification reasonably conveys that the Sig component is non-nucleotidyl. Furthermore, as explained below, I believe that any inference from the '997 specification and pending claims that the Sig component is or could be nucleotidyl in nature, or that Sig comprises a nucleotide, would be erroneous and unreasonable.

NONE OF THE EXAMPLES FOR THE SIG LABEL MOIETY ARE NUCLEOTIDYL OR A NUCLEOTIDE

10. At the outset I find it significant that the '997 specification discloses several examples for the Sig component -- all of which show that Sig is neither a nucleotide nor that it is nucleotidyl in its nature. Beginning on page 96, last paragraph, and continuing through the first paragraph on page 97, the '997 specification discloses:

> The Sig moiety employed in the make-up of the special nucleotides of this invention could comprise an ***enzyme or enzymic material***, such as ***alkaline phosphatase***, ***glucose oxidase***, ***horseradish peroxidase*** or ***ribonuclease***. The Sig moiety could also contain a ***fluorescing component***, such as ***fluorescein*** or ***rhodamine*** or ***dansyl***. If desired, the Sig moiety could include a ***magnetic component*** associated or attached thereto, such as a ***magnetic oxide*** or ***magnetic iron oxide***, which would make the nucleotide or polynucleotide containing such a magnetic-containing Sig moiety detectable by magnetic means. The Sig moiety might also include an ***electron dense component***, such as ***ferritin***, so as to be available by observation. The Sig moiety could also include a ***radioactive isotope component***, such as ***radioactive cobalt***, making the resulting nucleotide observable by radiation detecting means. The Sig moiety could also include a ***hapten component*** or per se be capable of complexing with an antibody specific thereto. Most usefully, the Sig moiety is a ***polysaccharide or oligosaccharide or monosaccharide***, which is capable of complexing with or being attached to a sugar or polysaccharide binding protein, such as a lectin, e.g. Concanavilin A. The Sig component or moiety of the special nucleotides in accordance with this invention could also include a ***chemiluminescent component***. [emphasis added]



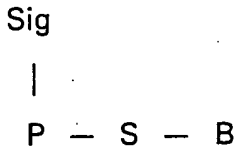
said Sig, when attached to said P moiety being capable of signalling itself or making itself self-detecting or its presence known and desirably the nucleotide is capable of being incorporated into a double-stranded polynucleotide, such as DNA, RNA or DNA-RNA hybrid and when so incorporated therein is still self-detecting. [emphasis added]

ii) Page 97, first full paragraph:

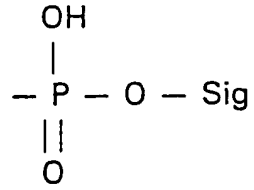
As indicated ***in accordance with the practices of this invention***, the ***Sig component*** could comprise any chemical moiety which is ***attachable*** either directly or through a chemical linkage or linker arm ***to the nucleotide***, such as ***to the [phosphate] P component*** (PM) thereof. [emphasis added]

iii) Originally filed claim 141:

141. A nucleotide having the general formula



wherein P is the phosphoric acid moiety, S the sugar moiety and B the base moiety, the phosphoric acid moiety being attached to the 3' and/or the 5' position of the sugar moiety when said nucleotide is deoxyribonucleotide and at the 2', 3' and/or 5' position when said nucleotide is a ribonucleotide, said base B being a purine or pyrimidine, said base B moiety being attached from the N1 or the N9 position to the 1' position of the sugar moiety when said base B is a pyrimidine or a purine, respectively, and wherein ***Sig is a chemical moiety is covalently attached to the phosphoric acid moiety*** via the chemical linkage



nucleotide" or to "the resulting nucleotide" (and the like). Some examples are provided below.

i) Page 95, lines 2-13:

The Sig chemical moiety is covalently attached to the phosphoric acid P moiety (PM) via the [phosphate] chemical linkage . . . said Sig, when attached to said P moiety (PM) being capable of signalling itself or making itself self-detecting or its presence known and desirably ***the nucleotide*** is capable of being incorporated into a double-stranded polynucleotide . . . [emphasis added]

ii) Page 96, lines 12-20

The chemical moiety Sig so attached to the nucleotide P-S-B (PM-SM-BASE) is capable of rendering or making the ***resulting nucleotide***, now comprising P-S-B (PM-SM-BASE) with the Sig moiety being attached to one or more of the other moieties, self-detecting or signalling itself or capable of making its presence known per se, when incorporated into a polynucleotide. . . [emphasis added]

iii) Page 96, lines 22-28

The Sig moiety desirably should not interfere with the capability of ***the nucleotide*** to form a double-stranded polynucleotide containing the ***special Sig-containing nucleotide*** in accordance with this invention and, when so incorporated therein, the ***Sig-containing nucleotide*** is capable of detection, localization or observation. [emphasis added]

iv) Page 99, lines 6-10

As indicated, such probes may contain one or more of the ***special Sig-containing nucleotides*** in accordance with this invention, preferably at least about ***one special nucleotide*** per 5-10 of the nucleotides in the probe. [emphasis added]

14. The just-quoted passages refer to the Sig-containing nucleotide as "the nucleotide," "the special Sig-containing nucleotide," "one special nucleotide," and "the resulting nucleotide," If the Sig component itself were a nucleotide, these passages would make little if any sense. There would be no "resulting nucleotide," rather, there would be a "resulting *di*nucleotide." Further, there would be no "one special nucleotide," but rather "a special *di*nucleotide." To illustrate this point more

a nucleotide (much like the sugar, phosphate and base moieties), and a nucleotide itself. A reading of the '997 specification clearly shows that Sig can only be a component of a nucleotide, and that it is not nucleotidyl or a nucleotide. Moreover, as explained earlier, Sig cannot be a nucleotide because it would force a person skilled in the art to define wrongly in several instances the word "nucleotide" in the '997 specification to mean or to refer to "a polynucleotide," "an oligonucleotide," or to "a dinucleotide."

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Sept. 3, 2004
Date

Dr. Alex A. Waldrop, III

* * * * * * *

FinalDecl.9.2.04(8PM)

Cell, Vol. 12, 23-36; September 1977, Copyrig 1977 by MIT

A Novel Method to Map Transcripts: Evidence for Homology between an Adenovirus mRNA and Discrete Multiple Regions of the Viral Genome

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Summary

A method has been devised which permits mapping of transcripts by a two-step hybridization procedure (sandwich hybridization). RNA extracted from cells infected with an adenovirus-SV40 hybrid (Ad2$^+$ND1) was hybridized to restriction endonuclease fragments of adenovirus type 2 (Ad2) DNA immobilized on nitrocellulose filters. RNAs containing both Ad2 and SV40 sequences formed duplexes through their Ad2 sequences, leaving their SV40 sequences as protruding tails. Annealing with ^{32}P-labeled SV40 DNA caused these tails to become labeled, permitting autoradiographic identification of the sequences of Ad2 DNA which are homologous to the RNA. The high sensitivity of this technique, achieved through the use of ^{32}P-labeled RNA of high specific activity, has led to the observation that hybridization of Ad2$^+$ND1 RNA occurs at several locations on the Ad2 genome, in addition to the expected sites of hybridization proximal to the SV40 insertion.

Introduction

Several approaches to mapping the regions of adenovirus type 2 (Ad2) DNA which are transcribed have been developed in recent years. These include saturation hybridization of unlabeled cytoplasmic RNA with the labeled, separated strands of restriction endonuclease fragments of Ad2 DNA (Flint, Gallimore and Sharp, 1975; Pettersson, Tibbetts and Philipson, 1976); hybridization of labeled cytoplasmic RNA of different size classes resolved by polyacrylamide gel electrophoresis to specific restriction endonuclease fragments (Tal et al., 1975; Craig, Zimmer and Raskas, 1975; Craig et al., 1975); hybridization of purified, labeled RNA to restriction endonuclease fragments of DNA transferred from agarose gels to nitrocellulose filters (Mathews, 1975; Söderlund et al., 1976); formation of RNA-DNA hybrids and their visualization in the electron microscope (Westphal, Meyer and Maizel, 1976; Chow et al., 1977b). These approaches have provided a detailed map of those adenovirus DNA sequences which serve as template for early and late viral gene transcription in cells productively infected with Ad2 (for recent review, see Flint, 1977).

We have developed a technique (sandwich hybridization) in which RNA is hybridized to defined fragments of viral DNA bound to nitrocellulose filters such that the 3′ or 5′ end of the RNA protrudes as a single-stranded tail. The DNA sequences complementary to the "tail" sequences can be determined by a second round of hybridization using specific fragments of viral DNA labeled with ^{32}P. By using DNA radioactively labeled to high specific activity in vitro as a probe, we have overcome some of the limitations to mapping RNA labeled in vivo.

As a model system, we have used RNA from cells productively infected with an Ad2-SV40 hybrid virus, Ad2$^+$ND1, originally isolated by Lewis et al. (1969). Ad2$^+$ND1 contains a 0.94 kilobase (kb) insertion of SV40 DNA which replaces 1.9 kb of the Ad2 genome located between positions 80.6 and 86 on the conventional physical map of Ad2 (see Figure 1A). The SV40 insertion comprises those sequences which lie between coordinates 11 and 28 on the circular map of SV40 DNA (Kelly and Lewis, 1973). At early times after infection with Ad2$^+$ND1, a limited portion of the SV40 sequences is expressed as cytoplasmic RNA. These early RNA sequences are complementary to the E strand of SV40 viral DNA (Khoury et al., 1973; Flint et al., 1975) and map between positions 28 and 17 on the physical map of SV40 DNA (Flint et al., 1975). All the SV40 E strand sequences in Ad2$^+$ND1 are expressed at late times during infection, but the RNA complementary to DNA sequences which map between positions 17 and 11 is much less abundant than that which is synthesized at exclusively early times (Khoury et al., 1973; Flint et al., 1975). Initiation of transcription does not seem to occur within SV40 DNA sequences integrated in the genome of Ad2$^+$ND1, and expression of SV40 DNA is believed to occur solely as a consequence of transcription initiated within Ad2 sequences (see Figure 1A).

An outline of the procedure we have used to map viral RNAs comprised of both SV40 and Ad2 sequences is shown in Figure 1B. Ad2 DNA is cleaved with a restriction endonuclease, the resulting fragments separated by agarose gel electrophoresis and transferred to a nitrocellulose filter (Southern, 1975). Unlabeled RNA extracted from cells lytically infected with an adenovirus-SV40 hybrid is hybridized to the immobilized DNA fragments, the filters are washed to remove unannealed RNA and hybridization is continued with SV40 DNA that has been labeled in vitro with ^{32}P to high specific activity (Kelly et al., 1970; Maniatis, Jeffrey and Kleid, 1975). After again washing the filters, the Ad2 DNA fragments complementary to RNA which contains SV40 sequences can be identified by autoradiography. We have in this way demonstrated the existence of an RNA molecule consisting of Ad2 and SV40 sequences in one continuous chain and have mapped the location of the sequences which serve as template for its synthesis on the Ad2$^+$ND1 ge-

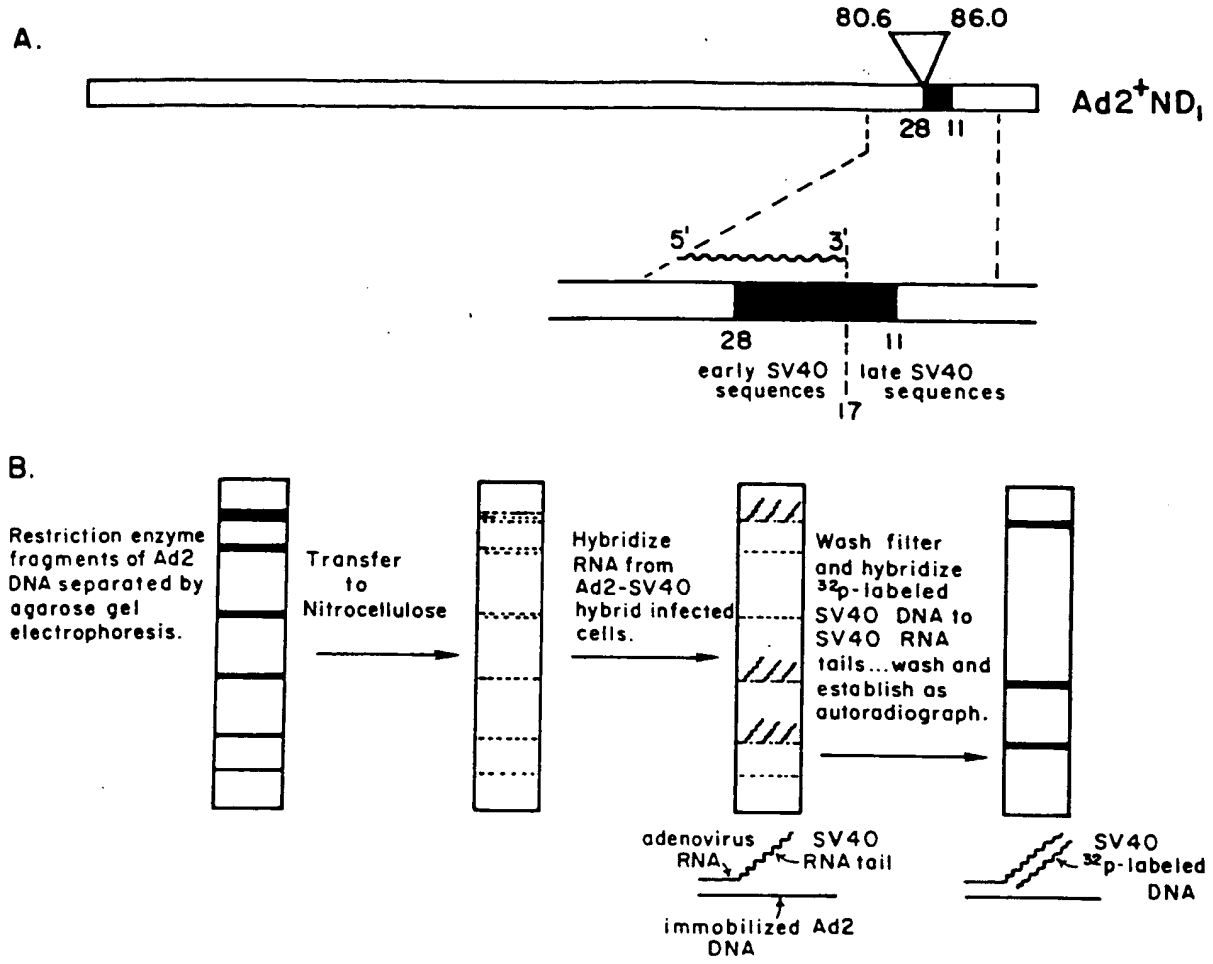


Figure 1. The Genome Structure of Ad2+ND1 (A) and a Schematic Representation of Sandwich Hybridization (B)

The DNA structure is taken from Kelly and Lewis (1973). Black areas represent the SV40 insertion showing the map positions on the SV40 viral genome. The coordinates of the Ad2 deletion in Ad2+ND1 are shown at the top of this figure. The proposed hybrid transcript is illustrated in the expanded part of this figure with its 5' end in Ad2 and its 3' end in SV40 sequences. Details of the sandwich hybridization procedure are given in the text.

nome. Moreover, the high level of sensitivity afforded by the sandwich hybridization technique has led to the unexpected observation that Ad2⁻ND1 RNA hybridizes not only to sequences adjacent to the SV40 insertion in Ad2⁺ND1, but also to other discrete regions of the Ad2 genome.

Results

Mapping of Cytoplasmic Hybrid Transcripts in Cells Infected with Ad2⁻ND1

To demonstrate the existence of a hybrid transcript in cells lytically infected with Ad2⁺ND1 and simultaneously to map its template, we have made use of several restriction endonucleases whose sites of cleavage on the SV40 and Ad2 genomes are well established. To determine the position of the 5' and 3' termini of this transcript on the Ad2⁻ND1 genome, Ad2 DNA was cleaved separately with Eco RI, Sma I, Hind III or Bgl II, and the resulting fragments were fractionated by electrophoresis through vertical slab gels cast with 1.0% agarose. The DNA fragments were then transferred directly to nitrocellulose filters (Southern, 1975) and used in hybridization experiments. Cytoplasmic RNA prepared from cells at late times (20 hr) after infection with Ad2⁻ND1 was annealed to viral DNA fragments. The hybridization filters were washed, and a second round of hybridization was carried out using ^{32}P-labeled SV40 DNA according to the scheme outlined in Figure 1B. The results of this experiment are shown in Figure 2.

Figure 2A is a photograph of the gel stained with ethidium bromide. The results of the hybridization experiment with late cytoplasmic Ad2⁻ND1 RNA are shown in Figure 2B. It is immediately clear that only a subset of those fragments shown in Figure 2A are

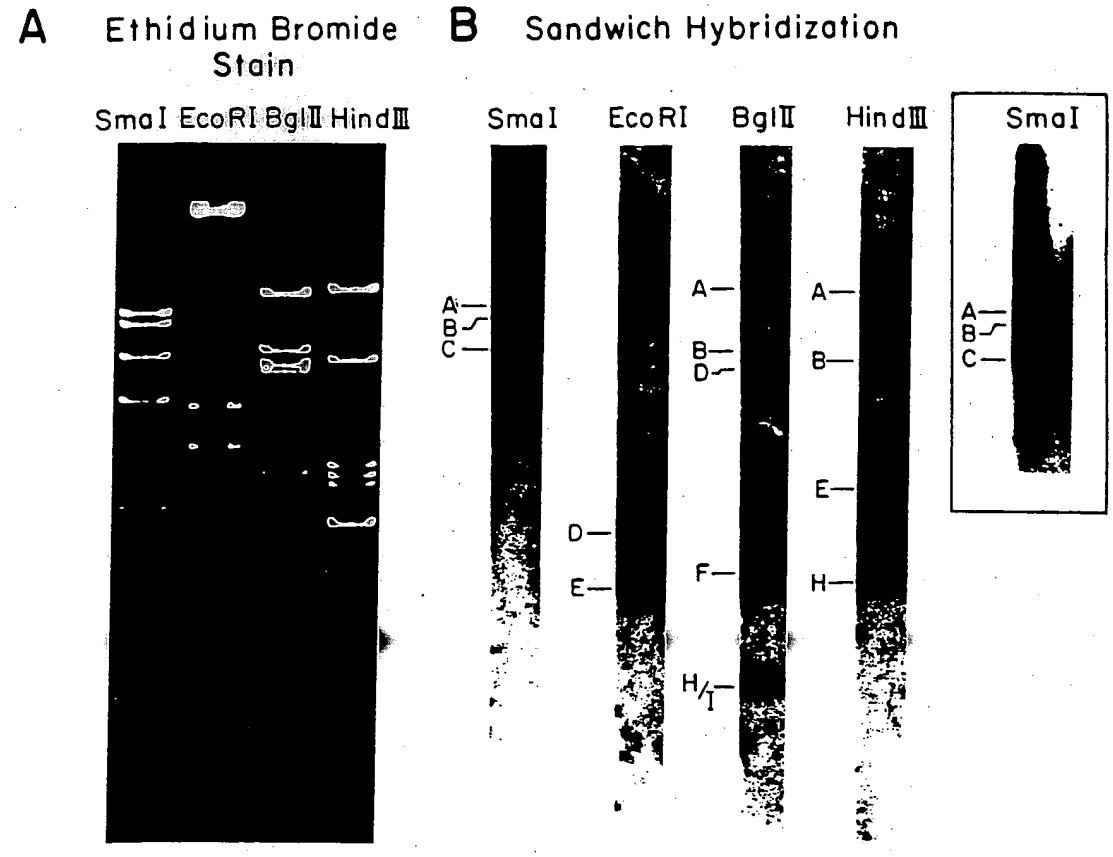
represented in Figure 2B. Fragments which map immediately adjacent to the SV40 insertion hybridize to the radioactive probe. These include Sma C, Eco RI-D and E, Bgl II-F and H or I (these fragments co-migrate in our gel system), and Hind H1-E and H. Because several restriction endonucleases have been used which generate a series of overlapping fragments whose map positions are known, it is possible to deduce the coordinates within which the RNA must be located. At late times (20 hr), the cytoplasmic transcript must map to the right of position 77.9 and extend into the SV40 insertion beginning at position 80.6. Ad2 sequences to the right of SV40 insertion represented by Eco RI-E, Bgl II-H or I, and Hind III-E also hybridize to the Ad2$^+$ND1 hybrid RNA. This hybridization may be due to RNA molecules which begin to the left of the SV40 insertion and continue through it into Ad2 sequences or to adenoviral DNA sequences that are believed to be repeated to the left and right of the SV40 insertion (L. Chow and T. Broker, personal communication). A map based on our analysis of these results is included in the bottom of Figure 2.

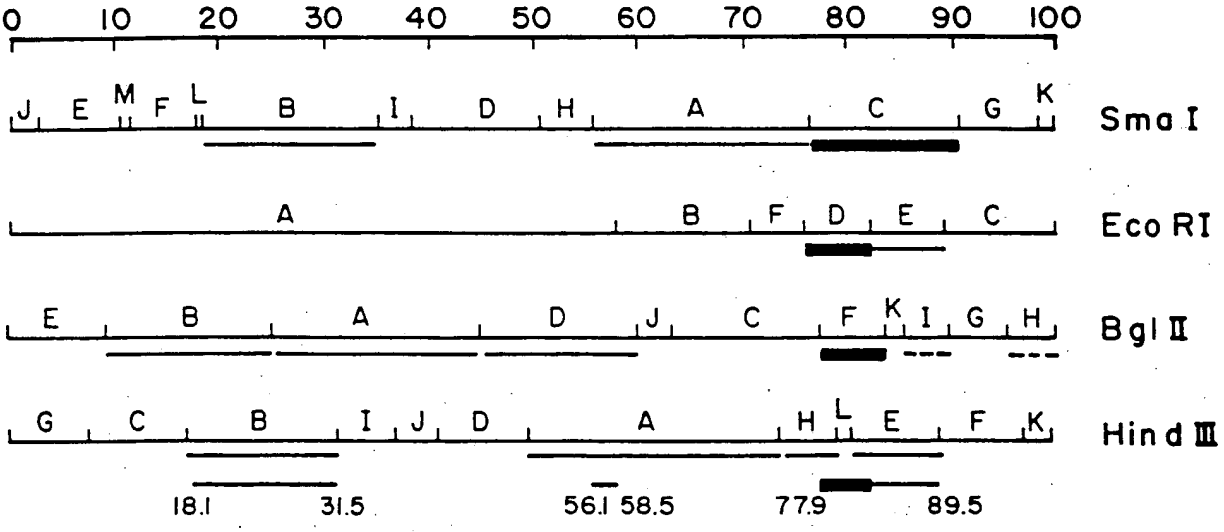
In addition to these sites of hybridization, fragments mapping distal to the SV40 insertion in Ad2$^+$ND1 also display hybridization. These fragments are Sma I-A and B, Bgl II-A, B and D, and Hind III-A and B. The significance of these observations is discussed below.

Control experiments have shown that if the RNA preparation is treated with pancreatic DNAase I (25 μg/ml for 30 min at 37°C), the hybridization pattern is unchanged; if the RNA is pretreated with pancreatic ribonuclease (25 μg/ml for 30 min at 37°C), all hybridization is completely abolished; if the nitrocellulose filters containing DNA fragments are treated with ribonuclease after the first round of hybridization with RNA, then no hybridization of ^{32}P-labeled SV40 DNA is observed to occur. These results strongly support the hypothesis that hybridization of the ^{32}P-labeled SV40 DNA probe is mediated by RNA molecules which contain both Ad2 and SV40 sequences in one continuous chain. The experiments reported here have been performed under conditions of DNA excess on the filter, because increasing the RNA concentration in the hybridization mixture results in an increase in hybridization of the ^{32}P-labeled probe to the immobilized DNA fragments. A detailed description of the sandwich hybridization method will appear elsewhere (A. R. Dunn and J. A. Hassell, manuscript in preparation).

One terminus of the hybrid RNA species found in the cytoplasm of Ad2$^-$ND1 infected cells maps somewhere between 77.9 and 80.6 on the conventional Ad2 map. To map the other end of the RNA, believed to be located within the SV40 insertion (Khoury et al., 1973; Flint et al., 1975), the Hind II and Hind III fragments of SV40 DNA were separately fractionated on agarose gels, transferred to nitrocellulose filters and annealed to late cytoplasmic RNA from Ad2$^+$ND1-infected cells. After washing, the filters were hybridized to ^{32}P-labeled Ad2 DNA to identify those SV40 sequences complementary to the hybrid RNA species. The results of this experiment are shown in Figure 3.

Figure 3A is a photograph of the gel stained with ethidium bromide. Figure 3B shows the results of the sandwich hybridization assay. SV40 Hind II fragment B or C and Hind III fragment A contain sequences homologous to the hybrid RNA. Because the SV40 sequences within Hind II-B are absent from Ad2$^+$ND1, we need not consider these further. No other fragments are complementary to the hybrid RNA (see Figure 3). The absence of hybridization to certain segments of the SV40 sequences contained within the genome of Ad2$^-$ND1 shows that the transcript terminates within the SV40 insertion. From the physical map of SV40 DNA (see Figure 3) and the hybridization data of Figure 2, it can be seen that the hybrid RNA maps no further left than position 77.9 on the Ad2 genome and no further right than position 17.5 within the SV40 insertion. Adenoviral sequences immediately to the left of the SV40 insertion in Ad2$^-$ND1 may also be repeated to the right of the SV40 insertion (L. Chow and T. Broker, personal communication). Because the late cytoplasmic Ad2$^-$ND1 RNA terminates within the SV40 insertion, we believe that the most probable explanation for the observed hybridization of Ad2 fragments to the right of the SV40 insertion (Figure 2) is due to these repeated sequences. We cannot, however, rule out the possibility that specific Ad2 mRNAs encoded by sequences to the right of the SV40 insertion become spliced to the hybrid RNA. It is possible to deduce from the DNA structure of Ad2$^-$ND1 and from the observation that only the E strand of SV40 viral DNA is transcribed in this hybrid (Khoury et al., 1973; Flint et al., 1975) that the r strand of adenoviral DNA must serve as template for the synthesis of the hybrid RNA. To confirm this, nitrocellulose filters containing the separated strands of the Ad2 Eco RI-D fragment were assayed for complementarity to the hybrid RNA species by sandwich hybridization. The results shown in Figure 4 clearly establish that the hybrid RNA anneals to the fast strand of Eco RI fragment D, which has previously been shown to share sequences with the r strand of intact viral DNA (Sharp, Gallimore and Flint, 1974). This establishes the direction of synthesis of the Ad2$^-$ND1 hybrid RNA species as being from left to right on the viral DNA molecule. In summary, the late cytoplasmic Ad2$^-$ND1 transcript


A
Ethidium Bromide Stain
SmaI EcoRI BglII HindIII
B
Sandwich Hybridization
SmaI
EcoRI
BglII
HindIII
SmaI
A
B
C
D
E
F
H/I


0 10 20 30 40 50 60 70 80 90 100
J E M F L B I D H A C G K
Sma I
A B F D E C
Eco RI
E B A D J C F K I G H
Bgl II
G C B I J D A H L E F K
Hind III
18.1 31.5
56.1 58.5
77.9 89.5

maps between 77.9 on the Ad2 genome and 17.5 on the SV40 insertion, and is transcribed from the viral r strand.

Mapping of Nuclear Hybrid Transcripts from Cells Infected with Ad2⁺ND1

High molecular weight viral RNA species larger than mRNA have been detected in cells infected with Ad2 (Parsons, Gardner and Green, 1971; McQuire, Swart and Hodge, 1972; Wall, Philipson and Darnell, 1972), and several lines of evidence favor a precursor-product relationship between large nuclear transcripts and mRNA (Bachenheimer and Darnell, 1975; Goldberg, Weber and Darnell, 1977; Weber, Jelinek and Darnell, 1977). We have made use of the sandwich hybridization technique to map hybrid transcripts present in the nucleus of cells lytically infected with Ad2⁺ND1. The results of such an experiment are shown in Figure 5. The overall picture is again one of selective hybridization (of the probe) to particular restriction endonuclease fragments. The most intense hybridization occurs to Sma I-C, Eco RI-D and E, Bgl II-F, and H or I (fragments H and I co-migrate in this gel system) and Hind III-E, which again represent the sequences adjacent to the SV40 insertion. The data summarized in the map which forms part of Figure 5 suggest that the hybrid transcript maps from position 77.9 on the Ad2 genome and extends beyond the Ad2-SV40 junction at position 86 but no farther than position 89.5 on the Ad2 genome. The ratio of the intensity of hybridization of Ad2⁺ND1 RNA to Ad2 Eco RI fragments D and E or Bgl II fragments I and F appears greater with nuclear RNA (Figure 5) than with cytoplasmic RNA (Figure 2). While in part this hybridization is due to the repeated Ad2 sequences located on each side of the SV40 insertion, it could also indicate transcription of SV40 sequences beyond position 17 terminating in Ad2 sequences. The latter explanation is consistent with the results of Flint et al. (1975), who have shown that at late times after infection, sequences complementary to the entire E strand of the SV40 insertion in Ad2⁺ND1 are present in nuclear RNA.

Evidence for Homology between Ad2⁺ND1 and Ad2⁺D2 RNA and Multiple Discrete Areas of the Viral Genome

Surprisingly, several bands representative of DNA fragments which do not map immediately adjacent to the SV40 insertion in Ad2⁺ND1 are apparent in Figure 2B. The fragments are invariably present and have been detected in six independent experiments: Sma I-A and B (see inset); Bgl II-A, B and D; Hind III-A and B. Because we have used overlapping restriction enzyme fragments, the transcripts which contain SV40 sequences can be shown to hybridize to two noncontiguous regions of Ad2 DNA which are located between positions 18.1–31.5 and 56.1–58.5 on the physical map (see Figure 2). With the exception of Eco RI-A, hybridization to a particular fragment in one restriction enzyme digest is borne out by hybridization to fragments in digests with other endonucleases occupying corresponding positions on the Ad2 genome. In many separate experiments, however, we have observed variability in hybridization of the probe to Eco RI-A. Restriction enzyme fragments which subdivide Eco RI-A, however, have always displayed hybridization to AD2⁺ND1 RNA (see Figures 2 and 5). The variability may be associated with the efficiency of transfer of DNA fragments from gels to nitrocellulose—a characteristic of high molecular weight DNA. Two restriction fragments, Bgl II-A and B (see Figure 2), are homologous to Ad2⁺ND1 RNA. This indicates either that there is one site of hybridization for the Ad2⁺ND1 RNA which spans the Bg1 II-A–B junction at position 25.3, or that there are a minimum of two sites within this region, one located in Bgl II-A and the other in Bgl II-B.

In Figure 5, we again observe hybridization of nuclear RNA to DNA sequences far distant from the SV40 insertion in AD2⁺ND1. The restriction enzyme fragments thus detected are, without exception,

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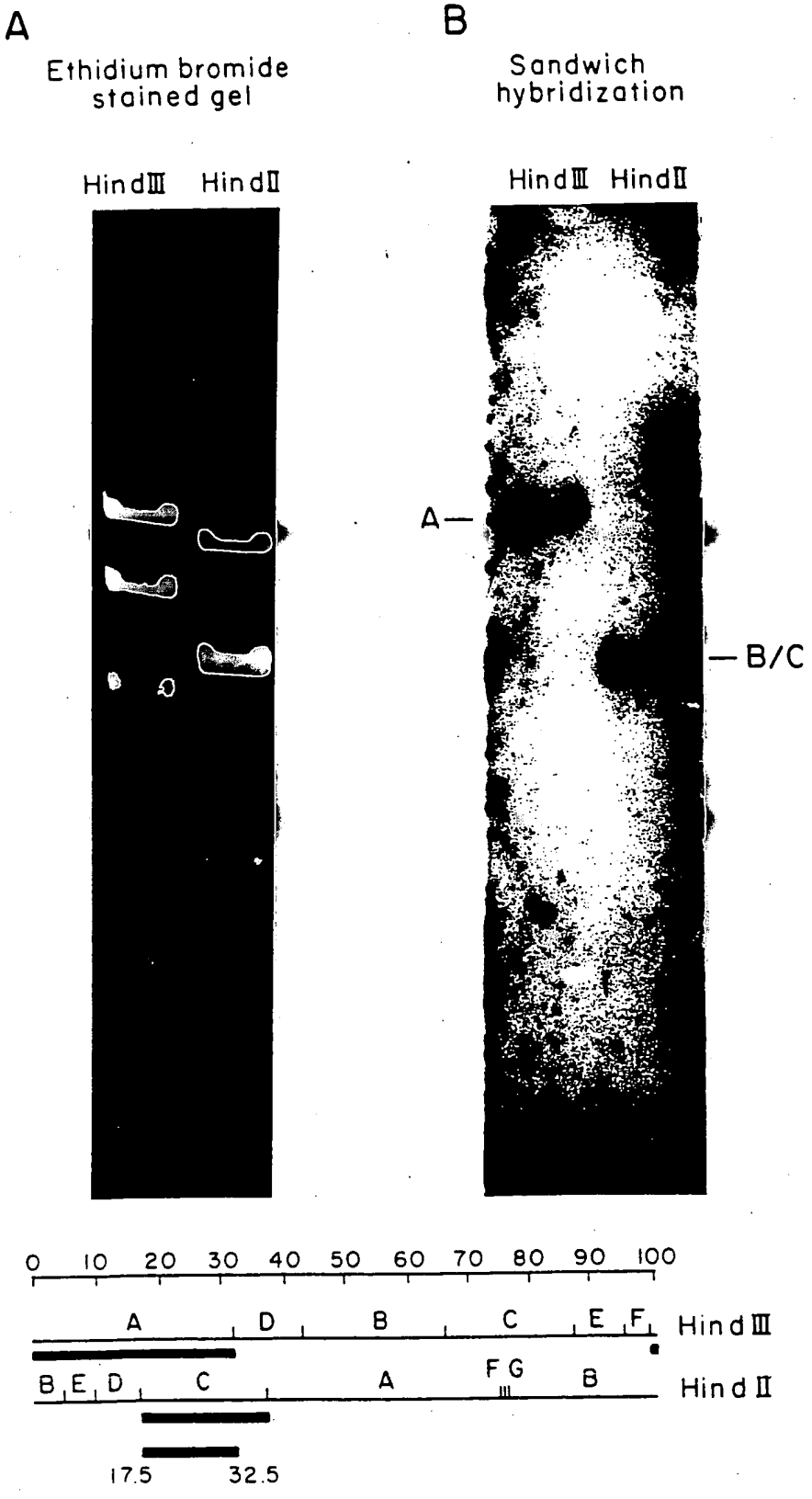
Figure 2. Analysis of Late (20 Hr) Cytoplasmic Hybrid RNA from Cells Infected with Ad2⁺ND1 Using Specific Fragments of Adenovirus DNA Immobilized on Filters

The products of digestion of Ad2 DNA with endonucleases Sma I, Eco RI, Bgl II and Hind III were fractionated by electrophoresis through 1% agarose gels for 12 hr at a potential of 1.5 V/cm and transferred to nitrocellulose as described in Experimental Procedures. Cytoplasmic poly(A)-containing RNA isolated from 2–5 × 10^8 cells infected with Ad2⁺ND1 at an moi of 5–10 PFU per cell was hybridized to a nitrocellulose filter containing viral DNA fragments. After washing, a second round of hybridization was carried out using 1 μg of ^{32}P-labeled SV40 DNA according to the scheme outlined in Figure 1 and detailed in Experimental Procedures. A sheet of Kodak XR-1 film inserted between two intensifying screens was placed over the nitrocellulose sheet and exposed for 3 days at −70°C. (A) represents the original ethidium bromide-stained gel; (B) is an autoradiograph showing sandwich hybridization. Two different exposures of our analysis using endonuclease Sma I are included. The autoradiograph shown in the inset was exposed for 8 days using No-Screen film. Bands which appear in the final autoradiograph have been identified by reference to the original ethidium bromide stained gel. p denotes the presence of a band representing hybridization to a partial digestion product.

Restriction enzyme maps showing the cleavage positions of endonucleases Sma I (Mulder et al., 1974), Eco RI (Pettersson et al., 1973), Hind III (Sambrook et al., 1975) and Bgl II (M. Zabeau, personal communication) are included in this figure. Thick and thin lines beneath fragments represent the presence of intense and relatively less intense autoradiographic bands, respectively. Dotted lines indicate the presence of a band corresponding to a fragment which cannot be distinguished from a fragment of similar molecular weight in our gel system. The coordinates of the hybrid RNA deduced from this analysis are displayed beneath the map.


A
Ethidium bromide stained gel
B
Sandwich hybridization
Hind III Hind II
Hind III Hind II
A—
— B/C
0 10 20 30 40 50 60 70 80 90 100
A D B C E F
Hind III
B E D C A F G B
Hind II
17.5
32.5

the same as those reported for cytoplasmic RNA (see Figure 2). We conclude that RNA sequences contained within the cytoplasmic and nuclear Ad2⁺ND1 hybrid RNAs are complementary to two discrete areas of the viral genome far removed from the SV40 insertion.

To determine whether this property was idiosyncratic of Ad2⁺ND1 RNA, we analyzed another adenovirus 2-SV40 hybrid, Ad2⁺D2, of entirely different structure (J. A. Hassell, E. Lukanidin, G. Fey and J. Sambrook, manuscript submitted) which also gives rise to hybrid RNAs. Ad2⁺D2 contains an insertion of SV40 DNA, 5 kb long, which comprises almost all the SV40 genome apart from those sequences between positions 54 and 63. A large segment of adenoviral sequences mapping between 76 and 96 is deleted from the Ad2⁺D2 genome (see Figure 6). To determine whether Ad2⁺D2 RNA hybridizes to genomic segments distal to the SV40 insertion, late cytoplasmic RNA from Ad2⁺D2-infected cells was annealed to the Eco RI and Hind III fragments of Ad2 DNA immobilized on nitrocellulose filters. Those fragments containing sequences complementary to hybrid mRNA were identified by hybridization to ^{32}P-labeled SV40 DNA. The results (Figure 6) show a familiar pattern. Fragments adjacent to the SV40 insertion display hybridization to ^{32}P-labeled SV40 DNA mediated by hybrid RNA. These include Eco RI-C and F, and Hind III-A, F or G, H and K. In addition, the hybrid mRNA species in Ad2⁺D2-infected cells also hybridize to Ad2 DNA sequences far removed from the SV40 insertion, Eco RI-A and Hind III-B. Interestingly, these are the same fragments which display hybridization to the Ad2⁺ND1 hybrid RNA (compare Figures 2, 5 and 7). Consideration of the structures of Ad2⁺ND1 and Ad2⁺D2 (together with the data presented in Figure 2) reveals that those Ad2 sequences which act as template for synthesis of the Ad2⁺ND1 hybrid RNA are completely deleted in Ad2⁺D2. Nevertheless, both hybrid transcripts anneal to DNA sequences within the same fragments (Eco RI-A and Hind III-B) of viral DNA, far removed from the SV40 insertion. This annealing is unrelated to the SV40 sequences which are shared between these hybrids because we could detect no homology between SV40 and Ad2 DNAs under the conditions of hybridization used in this work. Importantly, the Ad2⁺D2 transcript did not hybridize to Ad2 sequences adjacent to the SV40 insertion which serve as template for the transcription of Ad2⁻ND1 RNA. For example, it can be seen from Figure 6 that the Ad2⁻D2 hybrid RNA does not anneal with the Eco RI-D or Hind III-E fragments of Ad2 DNA. Similarly, the Ad2⁻ND1 tran-

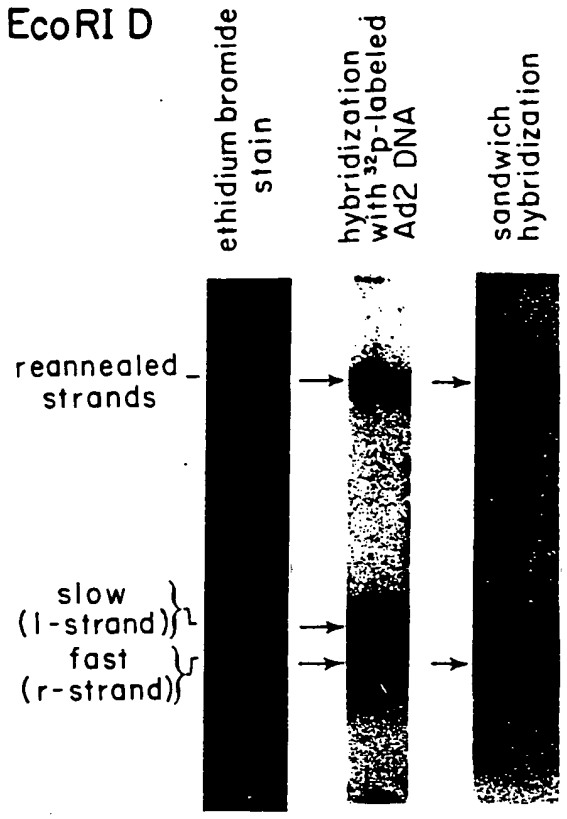


Figure 4. Sandwich Hybridization of Late (20 Hr) Cytoplasmic Ad2⁺ND1 RNA to the Separated Strands of Eco RI Fragment D

After cleavage of 20 μg of Ad2 DNA with endonuclease Eco RI, the products of digestion were separated by electrophoresis through preparative 1% agarose gels at a potential of 1.5 V/cm for 14 hr. Agarose strips containing fragment D were denatured in situ, and the strands were separated by gel electrophoresis through 1.4% agarose gels according to the general procedure described by Hayward (1972) and detailed in Experimental Procedures. After transfer to nitrocellulose, individual strips were each hybridized with late (20 hr) cytoplasmic polyadenylated RNA isolated from 2–4 × 10⁷ CV-1 cells infected with Ad2⁺ND1 at an moi of 5–10 PFU per cell. SV40 RNA tails were identified by hybridization with ^{32}P-labeled SV40 DNA. The autoradiograph was exposed for 3 days using No-Screen film. As a reference, one nitrocellulose strip was hybridized with 10^5 cpm of ^{32}P-labeled Ad2 DNA. Autoradiographic exposure time was 20 hr using No-Screen film.

Figure 3. Analysis of Late (20 Hr) Cytoplasmic Hybrid Transcripts Present in Cells Infected with Ad2⁺ND1 Using Specific Fragments of SV40 DNA Immobilized on Filters

The products of digestion of SV40 DNA with endonucleases Hind II and Hind III were fractionated by electrophoresis through 1% agarose gels for 12 hr at a potential of 1.5 V/cm and transferred to nitrocellulose. Cytoplasmic poly(A)-containing RNA isolated from 1–2 × 10^8 cells infected with Ad2⁺ND1 at an moi of 5–10 PFU per cell was hybridized to the nitrocellulose filters. About 1 μg of ^{32}P-labeled Ad2 DNA was hybridized to the protruding RNA tails, and the nitrocellulose filter was placed in contact with No-Screen film for a period of 6 days. (A) represents the original ethidium bromide stained gel; (B) is the sandwich hybridization. A map showing the genomic cleavage positions of endonucleases Hind III and Hind II (Danna, Sack and Nathans, 1973) and the fragments present in the sandwich hybridization autoradiograph is shown below.

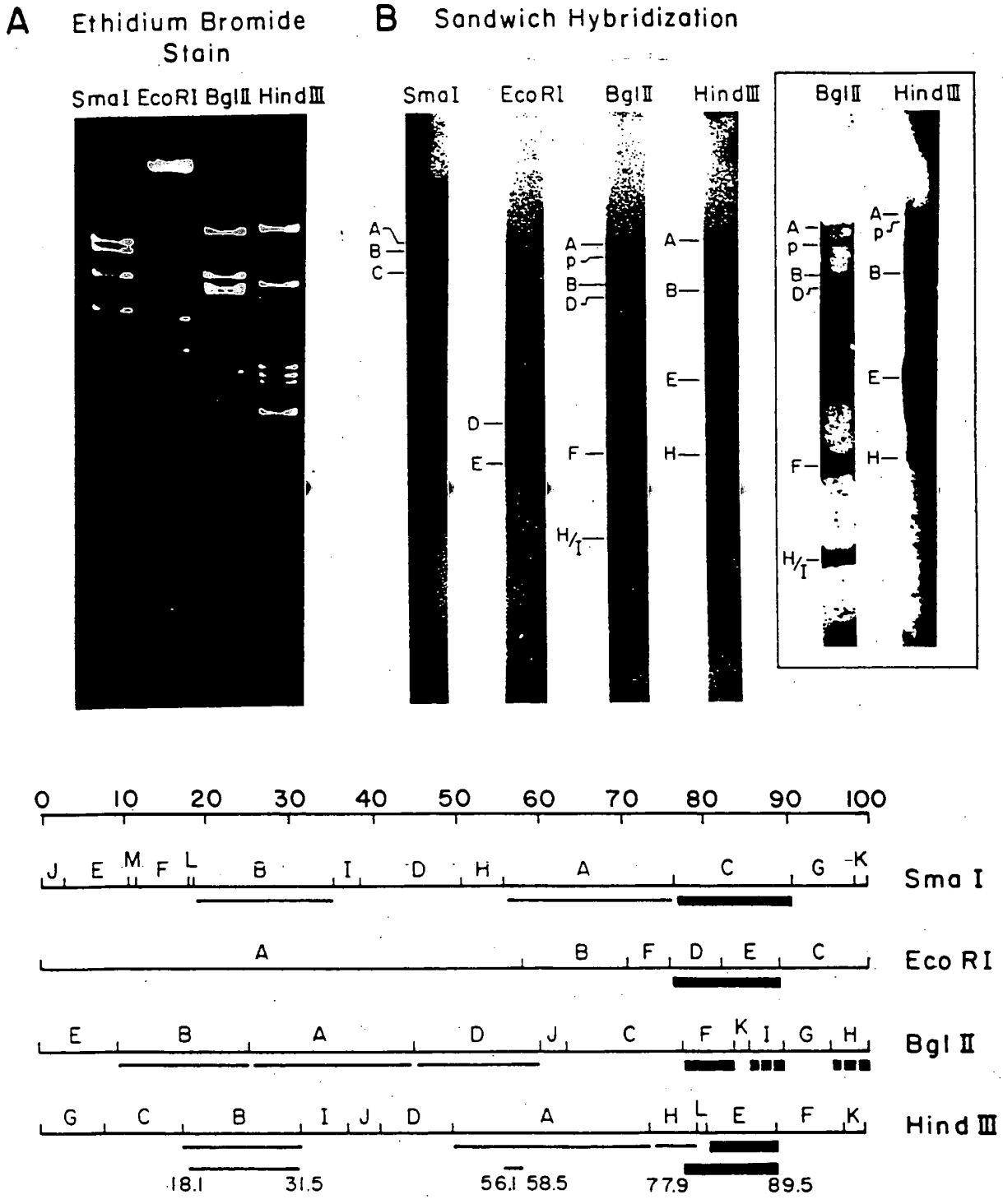


Figure 5. Analysis of Late (20 Hr) Nuclear RNA from Cells Infected with Ad2⁺ND1

Nuclear RNA isolated from 1–2 × 10⁶ CV-1 cells infected with Ad2⁺ND1 at an moi of 5–10 PFU per cell, and prepared as described in Experimental Procedures, was hybridized to restriction enzyme fragments of Ad2 DNA immobilized on a nitrocellulose filter prepared as described in the legend to Figure 2. After a second round of hybridization using ^{32}P-labeled SV40 DNA, the filter was placed in contact with No-Screen film and exposed for 8.5 days. Two high contrast prints of autoradiographs representing an analysis using Bgl II and Hind III are included as an inset in this figure. Autoradiographic exposure time using No-Screen film was 12 days. p denotes a band in the autoradiograph which represents hybridization to a partial enzyme product. A map summarizing the data from this analysis is included below the figure.

script does not hybridize to Ad2 sequences adjoining the SV40 insertion in Ad2+D2—that is, Eco RI-B and F (see Figure 2). From these experiments, we conclude that Ad2+ND1 and Ad2+D2 direct the synthesis of hybrid RNAs in which SV40 sequences are attached to Ad2 sequences adjacent to their respective SV40 insertions. Second, there is no detectable homology between the hybrid transcript of Ad2+ND1 and the Ad2 sequences which lie adjacent to the SV40 insertion of Ad2+D2. Conversely, the hybrid transcript of Ad2+D2 shows no detectable base sequence homology with the Ad2 sequences adjoining the SV40 insertion in Ad2+ND1 which encode the Ad2+ND1 hybrid transcript. Third, both hybrid RNAs share the property of annealing to the same regions of the Ad2 genome that are distant from the sites of insertion of SV40 sequences in Ad2+ND1 and Ad2+D2.

Discussion

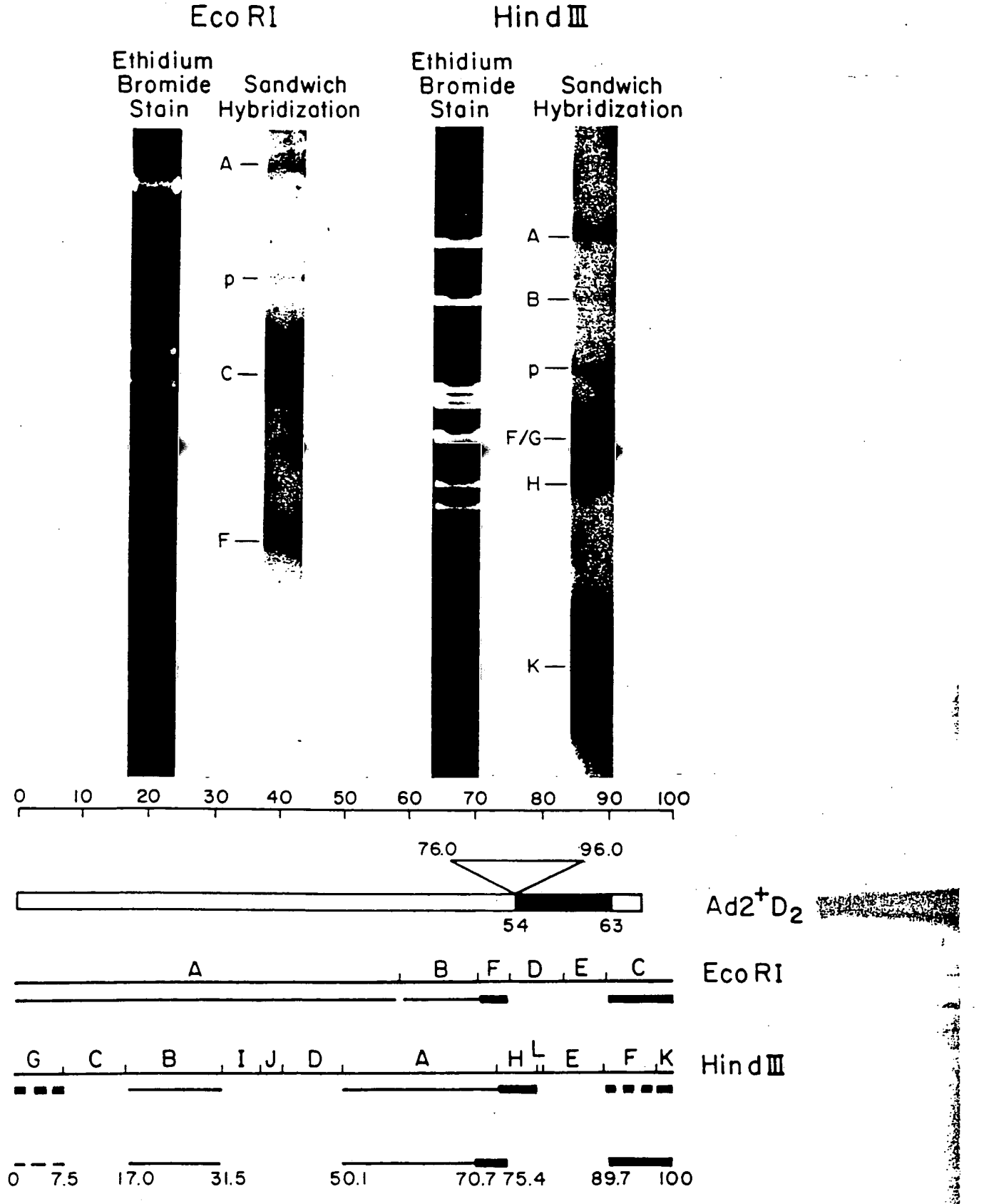
The sandwich hybridization method should be generally useful for mapping the chromosomal location of RNA transcripts, provided defined restriction enzyme fragments of DNA are available. The high sensitivity of the technique, achieved through the use of radioactive DNA of very high specific activity, should allow mapping of minor RNA species and of RNAs having homology with only a limited area of the genome. A particularly attractive application of the method is to map mRNAs terminating in poly(A). Because the template for poly(A) is not encoded within structural genes, ^{32}P-labeled oligo(dT) can be used as probe in hybridization mixtures containing defined fragments of DNA immobilized on nitrocellulose filters and poly(A)-containing RNA. We have not investigated all the factors which contribute to the sensitivity of the sandwich hybridization procedure. Clearly, the specific activity of the probe and the length of the RNA segment to which it is homologous are important. The most critical element, however, must be the intactness of the single-stranded RNA, which is especially difficult to maintain during prolonged periods of hybridization.

Using the sandwich hybridization technique, we have mapped the region within the Ad2+ND1 genome coding for RNA containing both Ad2 and SV40 sequences, and found unsuspected homology between this transcript and several discrete areas of the viral genome. Our interpretation of these results is summarized in Figure 7.

The late cytoplasmic Ad2+ND1 hybrid RNA, transcribed from the viral r strand, has its 5′ terminus located at approximately position 78 on the Ad2 genome and its 3′ terminus located at position 17 on the SV40 genome. The 5′ terminus of an early transcript maps at position 78.5 on the Ad2 genome (Chow et al., 1977b). Hence it seems very probable that the late Ad2+ND1 hybrid RNA, which is also expressed at early times (A. R. Dunn and J. A. Hassell, unpublished observations), is initiated or processed at this same site, rather than at a new "promoter" unique to Ad2+ND1. The template region of the Ad2+ND1 mRNA is large enough to code for a protein of molecular weight 49,000–53,000 daltons, yet the polypeptide specified by this region has a molecular weight of only 30,000 daltons (Lopez-Revilla and Walter, 1973; Grodzicker et al., 1974). Thus a large fraction of the mRNA for the 30,000 dalton protein must contain nontranslated sequences—a situation that seems to be common for several early Ad2 mRNAs (Lewis et al., 1976). Nuclear transcripts from Ad2+ND1-infected cells, in contrast to cytoplasmic RNA, hybridize more efficiently to DNA fragments mapping to the right of the SV40 insertion. Moreover, the entirety of the E strand sequences of the SV40 insertion in Ad2+ND1 is transcribed in the nucleus (Flint et al., 1975). Thus the cytoplasmic RNA may be derived from the nuclear transcript by cleavage and loss of 3′ terminal sequences. These results confirm and extend the mapping data of other investigators obtained by entirely different methods (Khoury et al., 1973; Flint et al., 1975).

The sensitivity of the sandwich hybridization technique has enabled us to detect homology between the Ad2+ND1 hybrid RNA and several areas of the viral genome distal to the SV40 insertion. Late cytoplasmic and nuclear Ad2+ND1 RNA is complementary to DNA sequences within the regions 18.1–31.5 and 56.1–58.5 on the Ad2 physical map, as well as to those sequences proximal to the SV40 insertion. One of these areas between 18.1 and 31.5 contains sequences known to encode the VA species of RNA, but few, if any, other individual RNA species have been mapped here (Mathews, 1975; Flint, 1977). The second major area of homology between the Ad2+ND1 hybrid RNA species and the viral genome maps between coordinates 56.1 and 58.5. R loop mapping has placed an mRNA, probably that coding for the hexon polypeptide (Lewis et al., 1975; Westphal et al., 1976; Chow et al., 1977b), between coordinates 52 and 62 on the physical map of Ad2. The hybrid species must therefore either share sequences with hexon mRNA or contain sequences complementary to this mRNA.

Many other adenovirus 2-SV40 hybrid viruses give rise to mRNAs containing adenoviral and SV40 sequences in one continuous chain. A comparison between the hybrid RNAs induced by Ad2+ND1 and Ad2+D2 reveals that there is no detectable homology between the Ad2+ND1 hybrid transcript and the


Eco RI
Hind III
Ethidium Bromide Stain
Sandwich Hybridization
Ethidium Bromide Stain
Sandwich Hybridization
A
p
C
F
A
B
p
F/G
H
K
0 10 20 30 40 50 60 70 80 90 100
76.0
96.0
54
63
Ad2+D2
A B F D E C
Eco RI
G C B I J D A H L E F K
Hind III
0 7.5 17.0 31.5 50.1 70.7 75.4 89.7 100

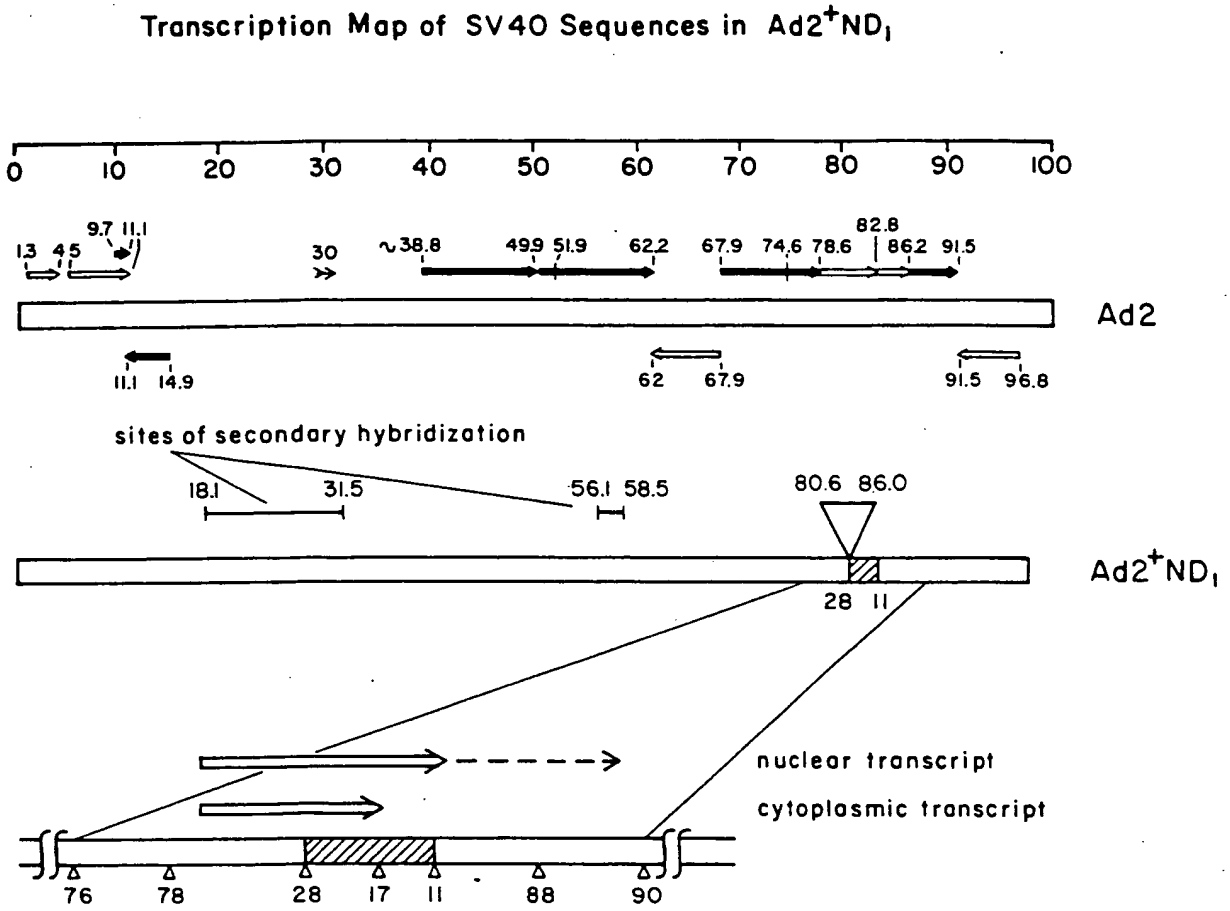


Figure 7. Transcription Map of SV40 Sequences in Ad2+ND1

The adenovirus genome is shown on the top scale, 0–100 units. A transcription map of adenovirus 2 determined by electron microscopy of RNA:DNA hybrids is taken from Chow et al. (1977b). The arrows above and below the Ad2 genome designate the locations and polarities of early (open arrows) and late (closed arrows) cytoplasmic RNA. The thin double arrow represents the approximate location of the VA species of RNA (Mathews, 1975; Söderlund et al., 1976). The regions of secondary hybridization for the Ad2+ND1 hybrid RNA are indicated above the map of the Ad2+ND1 genome. Our interpretation of the sandwich hybridization results are presented in the exploded view of the Ad2+ND1 genome. The open arrows represent the position and polarity of the Ad2+ND1 hybrid species. The dotted line designates the region within which the nuclear transcript must terminate.

adenoviral sequences which lie adjacent to the SV40 insertion of Ad2+D2, and conversely, the Ad2+D2 hybrid transcript shows no homology with the Ad2 sequences adjoining the SV40 insertion in Ad2+ND1. Both hybrid RNAs, however, are complementary to adenoviral sequences that map within the same region of the Ad2 genome distal to the SV40 insertion in Ad2+ND1 and Ad2+D2. The Ad2+ND1 and Ad2+D2 hybrid RNA may therefore contain different adenoviral sequences which are repeated elsewhere in the Ad2 genome, or they may contain common adenoviral sequences which are not encoded within adenoviral sequences adjacent to their respective SV40 insertions, but encoded at a separate discrete location on the Ad2 genome. The latter possibility is consistent with several recent observations. First, adenovirus 2 DNA fragments which encompass the region be-

Figure 6. Analysis of Late (20 Hr) Cytoplasmic Hybrid RNA from Cells Infected with Ad2+D2

Late cytoplasmic polyadenylated RNA was isolated from 0.5–1.0 × 10^8 CV-1 cells infected with Ad2+D2 at an moi of 5–10 PFU per cell as described in Experimental Procedures, and hybridized to nitrocellulose filters containing restriction enzyme fragments of Ad2 DNA generated by cleavage with endonucleases Eco RI and Hind III (see legend to Figure 2). RNA tails were identified using 1 μg of ^{32}P-labeled SV40 DNA. Autoradiographic exposure time was 12 hr using No-Screen film. p denotes the presence of a band representing hybridization to a partial digestion product.

A map showing the structure of Ad2+D2 and the cleavage positions of endonucleases Eco RI and Hind III on Ad2 DNA is included in this figure. Thick and thin lines represent the presence of intense and relatively less intense bands. The broken line corresponds to a fragment which cannot be distinguished from a fragment of similar molecular weight in our gel system.

tween 17 and 32 on the genome select, by hybridization in solution, all but two of the major late structural gene transcripts of the virus (Lewis, Anderson and Atkins, 1977). Second, the 5′ end of hexon mRNA (S. M. Berget, C. Moore and P. A. Sharp, manuscript submitted) and several other late adenovirus 2 mRNAs (Chow et al., 1977a) do not base-pair with the adjacent viral DNA sequences in R loop structures, but do hybridize with viral sequences which map at positions 16.7, 19.7 and 26.7 (Chow et al., 1977a). Finally, two of the most abundant Ad2 mRNAs (100K and fiber), isolated late during infection, have a common capped T1 ribonuclease-resistant oligonucleotide at their 5′ ends which is encoded between positions 14.7 and 17.0 on the physical map of Ad2 and not by DNA sequences adjacent to their structural genes (Klessig, 1977). While the observed hybridization of the Ad2⁻ND1 and Ad2⁻D2 RNAs to positions 18.5–31.5 may be accounted for by RNA sequences encoded by DNA sequences between 18.5 and 31.5, which are added post-transcriptionally to the hybrid RNA species (Chow et al., 1977a), the nature of the hybridization to position 56.1–58.5 remains enigmatic.

Several mechanisms can be imagined by which two or more RNA chains, derived from noncontiguous regions of the viral genome, become joined. First, independent RNA chains may be joined by an RNA ligase to yield a complete mRNA. Second, small RNAs coded within one area of the viral genome may act as primers for mRNA elongation at other sites on the viral genome (Dickson and Robertson, 1976). Third, a large precursor to mRNA may assume a conformation, imposed by intramolecular hydrogen bonding or by hydrogen bonding to small RNAs, to bring together sequences derived from distal regions of the viral genome such that endoribonuclease cleavage followed by RNA ligation places these end to end (Klessig, 1977). Fourth, the template for mRNA synthesis may assume a specific conformation such that noncontiguous areas become juxtaposed during transcription. RNA polymerase may "jump" from one segment to the other, carrying nascent RNA chains with it until it reaches a termination signal and is released from the template. Finally, the viral genome may rearrange by recombination to yield new templates for mRNA synthesis. So far there is no information which favors any of these models over any other.

Whatever the mechanism, the conclusion seems unavoidable that RNA sequences derived from multiple noncontiguous areas of the viral genome become covalently linked during or after transcription. Definitive proof that this occurs can be obtained by primary sequence data of specific mRNAs and the DNA segments encoding them.

Experimental Procedures

Cells and Viruses

CV-1 cells, a line of African green monkey kidney cells (Jensen et al., 1964), were cultured in plastic dishes in Dulbecco's modified Eagle's medium (Dulbecco and Freeman, 1959) supplemented with fetal bovine serum (5% v/v), streptomycin (100 units per ml) and penicillin (100 units per ml). Human HeLa cells (Gey, Coffman and Kubicek, 1952) were grown as suspension cultures in F13 medium (F13; Gibco, New York) supplemented with horse serum (5% v/v) and antibodies at the concentrations specified above.

Adenovirus type 2 and Ad2⁺ND1 were propagated in suspension cultures of HeLa cells (Pettersson, Philipson and Hoglund, 1967). Virions were purified from infected cells by the method of Green and Pina (1963) as modified by Lonberg-Holm and Philipson (1969). SV40 (strain 777) was grown in CV-1 cells at low multiplicity of infection (>0.01 PFU per cell) from a twice-plaque-purified stock.

Isolation of Viral DNA

The DNA of adenovirus type 2 was isolated from purified virions as described by Pettersson and Sambrook (1973). SV40 DNA was isolated 48–72 hr post-infection from CV-1 cells infected at a multiplicity of 3–6 PFU per cell by the method of Hirt (1967).

Isolation of RNA

Cytoplasmic extracts were prepared from CV-1 cells infected with Ad2⁺ND1 at a multiplicity of 5–10 PFU per cell. The medium was aspirated from infected cultures, and after rinsing the monolayers with ice-cold phosphate-buffered saline (PBS), the cells were scraped into PBS and concentrated by centrifugation. Cytoplasmic extracts were prepared by resuspending cells in isotonic buffer [0.15 M NaCl; 0.01 M Tris-HCl (pH 7.6), 0.0015 M $MgCl_2$] and treating with 0.5% Nonidet P40 (NP40). Purification of cytoplasmic RNA was carried out as described by Craig and Raskas (1974). Nuclei were washed in isotonic buffer containing 0.5% NP40, 0.5% sodium deoxycholate (Penman, 1966) and RNA prepared according to the method of Craig and Raskas (1974).

Oligo(dT)-Cellulose Chromatography

Isolation of polyadenylated RNA molecules was carried out by chromatography using oligo(dT)-cellulose (Collaborative Research, Inc.). Total cytoplasmic RNA was applied to a 1 ml column of oligo(dT)-cellulose in 2 ml of loading buffer containing 0.5 M NaCl, 10 mM Tris (pH 7.6). The flow through was applied 3 times, after which the column was washed through with 10 ml of loading buffer. Poly(A)-containing molecules were eluted from the column using 10 mM Tris (pH 7.6). The salt concentration of the solution was adjusted to 0.15 M NaCl, and the RNA precipitated by addition of 2 volumes of ethanol at −20°C.

Radioactive Labeling of Ad2 and SV40 DNA

Ad2 and SV40 DNA were labeled in vitro with α^{32}P-nucleotides by the nick translation reaction of E. coli polymerase I (Kelly et al., 1970) using the technique developed by P. Berg and his colleagues (Rigby et al., 1977), and the conditions established by Maniatis et al. (1975). DNA polymerase I was purchased from Boehringer Mannheim Biochemicals. The reaction was stopped by the addition of SDS and EDTA to final concentrations of 0.5% and 0.01 M respectively. After two extractions with phenol the unincorporated deoxynucleotide triphosphates were separated from the radioactive DNA by passage over a G-50 sephadex column equilibrated with 10 mM Tris (pH 7.8); 2 mM EDTA. The specific activity of the product was in the range of 0.5 –1 × 10^8 cpm/μg.

Restriction Endonucleases

Endonuclease Hind II and Hind III were isolated from Hemophilus influenzae serotype d (Smith and Wilcox, 1970) as described by Lai and Nathans (1974). Endonuclease Sma I was isolated from Seratia marcescens by an unpublished method of R. Greene and

C. Mulder. Eco RI was isolated from E. coli and purified as described by Yoshimori (1971). Endonuclease Bgl II was isolated from Bacillus globigii by an unpublished procedure of G. A. Wilson and F. E. Young. Reaction mixtures for restriction endonucleases Hind II, Hind III and Bgl II contained 0.01 M Tris-HCl (pH 7.7), 0.01 M $MgCl_2$, and 0.001 M dithiothreitol (DTT). Endonuclease digestions with Sma I contained 0.03 M Tris-HCl (pH 9.0), 0.01 M $MgCl_2$, 0.001 M DTT, 0.01 M KCl, and those with Eco RI contained 0.09 M Tris- HCl, 0.01 M $MgCl_2$, 0.001 M DTT. Digestions were performed at 37°C, except for Sma I which was carried out at 30°C. The incubation times varied depending upon the enzyme used. Reactions were stopped by the addition of EDTA to a final concentration of 0.01 M.

Agarose Gel Electrophoresis

Gel electrophoresis of DNA samples was performed in vertical slab gels (Studier, 1973) 17 × 15 × 0.4 cm cast with 1.0% agarose as described by Sugden et al. (1975). At the completion of electrophoresis, the DNA within the gels was visualized by ultraviolet illumination after staining with ethidium bromide (Sharp, Sugden and Sambrook, 1973). The gels were photographed using a Kodak 22A filter and Tri-X film.

Separation of Viral DNA Strands

The procedure is a modification of an original method described by Hayward (1972) and more recently by Sharp et al. (1974). Fragments of Ad2 DNA generated by cleavage with Eco RI were separated by electrophoresis through 1% agarose gels. Strips of agarose containing specific fragments were cut from the gels, and the DNA was denatured in situ by immersing in 0.3 N NaOH for 30 min at room temperature. After rinsing for 15 min in several changes of distilled water, agarose strips were loaded on performed gels cast with 1.4% agarose such that the length of one side of the strip was in direct contact with the surface of the gel. Electrophoresis was carried out at a potential of 1.5 V/cm for 18 hrs in phosphate buffer (Hayward, 1972). Separated strands of DNA were visualized by staining with ethidium bromide (0.5 μg/ml in electrophoresis buffer). The separated strands were then transferred to nitrocellulose filters as described below.

Blotting

Gels containing DNA fragments were submersed in 0.2 M NaOH, 0.6 M NaCl for 45 min at room temperature. After rinsing in distilled water, the gels were transferred to a bath containing 1.0 M Tris-HCl (pH 7.4), 0.6 M NaCl. DNA was transferred onto a sheet of nitrocellulose (B6, Schleicher and Schüll), essentially using the method of Southern (1975). DNA was immobilized by incubation of nitrocellulose filters at 80°C for 2 hr in a vacuum oven.

Hybridization

Hybridizations were performed essentially as described by Botchan, Topp and Sambrook (1976). Prior to hybridization, the nitrocellulose filters were incubated for 6 hr at 65°C in a solution containing 6 × SSC, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin (Denhardt, 1966).

After ethanol precipitation of nuclear and polyadenylated cytoplasmic RNA, the pellets were dissolved in a small volume of 10 mM Tris (pH 7.6), 2 mM EDTA. Hybridization was carried out in a final volume of 3 ml containing 6 × SSC, 0.5% SDS, 2 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin. Nitrocellulose filters were rolled into cylinders and inserted into 150 × 25 mm test tubes. Hybridization fluid was added; the tubes were sealed with Teflon stoppers and tape, and set in a horizontal position on a rotating wheel submersed in a water bath at 65°C. Following overnight hybridization (12-16 hrs), the filters were exhaustively washed in 2 × SSC, 0.5% SDS for 6 hrs at 65°C. At the end of this period, the washing solution was replaced with 3 ml of hybridization fluid containing 1 μg denatured ^{32}P-labeled SV40 or Ad2 DNA (spec. act. 5×10^7–1×10^8 cpm/μg), 6 × SSC, 0.5% SDS, 2 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin. Hybridization was carried out at 65°C for 12-16 hr, after which filters were exhaustively washed in 2 × SSC, 0.5% SDS for 6-8 hr at 65°C. After rinsing in 2 × SSC, filters were air-dried, mounted on Whatman 3M paper and established as autoradiographs.

Autoradiography

Nitrocellulose filters were placed in direct contact with No-Screen film, type NS-54T (Kodak), for periods of time ranging from 24 hrs to 3 weeks. In some cases, where the autoradiographic signal was weak, use was made of one or two intensifying screens (Cronex, Lightening Plus, DuPont) and Kodak XR-1 Film. Exposure was carried out at −70°C.

Acknowledgments

We thank Joe Sambrook for suggesting the sandwich hybridization assay, critical readings of the manuscript and many useful discussions; Mike Botchan and Mike Mathews for helpful discussions; Rich Roberts for his comments on the manuscript; Ronni Greene for providing restriction endonucleases; Steve Young and Bob McGuirk for their technical assistance; Bob Yaffe and Ted Lukralle for the illustrations; and Joyce Schneider for typing the manuscript. This research was funded by a grant from the National Cancer Institute. J.A.H. is supported by a National Research Service Award.

Received June 9, 1977; revised July 5, 1977.

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Methacrylate Polymerization by AzoRNA: Potential Usefulness for Chromosomal Localization of Genes

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Synopsis

An azo pyrimidine nucleotide has been prepared and enzymatically attached to oligo(A) primers. The nucleotide's azo pyrimidine group has previously been shown to initiate polymerization of methacrylate esters designed to bind marker groups for visualization by microscopy. When attached to RNA molecules complementary to a chromosomal DNA segment, these nucleotides may allow localization of the DNA segment following *in situ* hybridization of the probe, methacrylate polymerization, and marker attachment. Since mRNA molecules of potential interest as probes bear a 3′-poly(A) tail, the modified nucleotides were added to oligo(A) primers as models. First, N^4-ureidocytosine nucleotides were enzymatically added to ApApA, $(Ap)_9A$, or [5′-^{32}P]-$(pA)_{10}$, using the modified cytidine 5′-diphosphate and "primer-dependent" polynucleotide phosphorylase (*M. luteus*). In the case of the ApApA-primed reaction, the N^4-ureidocytosine nucleotides in the product polynucleotide were converted into azo nucleotides by oxidation with *N*-bromosuccinimide. The other two primers were employed to study the time course of polynucleotide formation and to verify that primer was indeed being utilized by the enzyme. The suitability of the modified nucleotide for *in situ* hybridization studies was examined. Poly(N^4-ureidocytidylic acid) was prepared from poly(C) and semicarbazide by the bisulfite-catalyzed transamination reaction. It was found that 95% of the N^4-ureidocytosine nucleotides in this polynucleotide survive the elevated temperatures typically required for DNA:DNA denaturation and RNA:DNA annealing. When poly(N^4-ureidocytidylic acid) was mixed with poly(I) in buffered aqueous salt solutions, no evidence for hybridization was found, so binding of the probe RNA to the denatured chromosomal DNA molecule via the modified nucleotides is not expected. Upon oxidation of poly(N^4-ureidocytidylic acid) with *N*-bromosuccinimide, the azo nucleotides were formed, as judged by the appearance of a characteristic peak at approximately 350 nm in the uv-absorption spectrum of the yellow-orange product, azoRNA. The azo nucleotides in azoRNA exhibited the expected acid lability, which is known to be accompanied by 1-glyceryl methacrylate polymerization in the case of the simple azo pyrimidine. Because 1-glyceryl methacrylate bears substituent glycol groups for attaching heavy atoms or fluorescent markers, it is possible that probe RNA molecules bearing azo nucleotides may be useful for localizing low-multiplicity genes along eukaryotic chromosomes.

* To whom correspondence should be addressed. Abbreviations: azoRNA, RNA containing 4-aminocarbonylazo-2-pyrimidinone nucleotides; CDP, cytidine 5′-diphosphate; u^4C, N^4-ureidocytosine.

Biopolymers, Vol. 20, 2635–2648 (1981)

CCC 0006-3525/81/122635-14$01.40

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INTRODUCTION

In situ RNA·DNA hybridization is a powerful method for localizing genes along eukaryotic chromosomes.[1] In this method the chromosomal, double-strand DNA molecule is denatured by treatment with alkali, hot formamide, or urea. Then radiolabeled RNA that is complementary to a particular DNA segment is allowed to anneal *in situ* to the chromosomal DNA. After removal of nonspecifically bound RNA by washing or treatment with RNase, the specimen is coated with photographic emulsion to reveal the sites that have bound RNA. Probes that have been used include mRNA,[2] rRNA,[3] and tRNA.[4] Examples of the use of this method include the localization of the histone genes on the long arm of human chromosome 7 and the 5S rRNA genes on the long arm of chromosome 1.

A major strength of this method is the relatively high resolution achievable, a particular DNA sequence often being assigned to a single chromosomal band. Also of importance is the potential to localize DNA segments that are not genetically expressed if the appropriate RNA probe is available using *in vitro* methods.[5]

Currently, the method is limited to the study of reiterated nucleotide sequences, such as satellites or tandem repeats of genes in ordinary (mononeme) chromosomes and single-copy genes in polytene chromosomes. The limited applicability of this method arises from the limited efficiency of RNA annealing to specimen DNA,[6,7] nonspecific binding of the probe to the specimen,[8] and from the limited specific activity of radiolabeled RNA that is achievable.[1,9] The efficiency problem has been largely surmounted by the use of excess RNA to drive the reaction and by careful selection of the optimum temperature for carrying out the annealing.[6,10] The nonspecific binding of the probe, which results in background silver grain production, has been largely overcome by the use of aqueous formamide hybridization solutions[11,12] and by pretreatment of the specimen[13] and glassware.[10]

An approach for circumventing the limitation on the specific activity of RNA that is achievable was recently reported. A mixed RNA–DNA probe composed of a viral nucleotide sequence (RNA) attached to a long, radiolabeled amphibian DNA has reportedly led to the localization of single-copy viral sequences in the chicken genome.[14] The long piece of amphibian DNA was required for incorporation of sufficient radiolabel to allow subsequent autoradiographic detection of the probe. This method suffers from the limitation, however, that the RNA–DNA probe molecule is actually longer than several of the human chromosomes, so direct assignment of genes to specific chromosomal regions is not possible. Because there may be approximately 50,000 structural genes in man present as only single-copy sequences,[15] it would be desirable to increase the sensitivity of the *in situ* hybridization method without sacrificing resolution.

To increase the sensitivity, we have devised an alternative to autoradiography for depositing metal in the vicinity of the RNA probe. In this approach, a string of modified nucleotides is added to the RNA probe and

then the probe is hybridized to its chromosomal DNA complement. The modified nucleotides are designed to initiate polymerization of a methacrylate monomer that is capable of binding marker groups (fluorescent or heavy-atom-containing groups). Polymerization of the methacrylate in the vicinity of the modified nucleotides generates chains of methacrylate units to which marker groups are then attached. The markers are subsequently localized by fluorescence or electron microscopy. In essence, radioactive decay and photographic development are replaced by generation of a methacrylate polymer that binds many marker groups.

We have developed an azo pyrimidine that initiates methacrylate polymerization in mildly acidic aqueous solution.[16] Polymerization of 1-glyceryl methacrylate yielded a polymer with pendant glycol groups for attachment of many fluorescent groups or Os(VI) atoms.

We report here the attachment of the azo pyrimidine nucleotides to oligomeric RNA primers and the assessment of the potential utility of this type of probe for *in situ* hybridization studies.

EXPERIMENTAL

Ultraviolet-absorption spectra were recorded on a Perkin-Elmer 552 spectrophotometer. Measurements of pH were accomplished with a Radiometer PHM 62 pH meter. "Primer-dependent" polynucleotide phosphorylase (*Micrococcus luteus*) was from P-L Biochemicals. Plastic tubes were used for enzyme reactions. *N*-Bromosuccinimide was from Matheson, Coleman, and Bell. Acrylamide was from Bio-Rad Labs. Poly(C) was from Miles. DEAE Sephadex A-25, DEAE cellulose, and CDP (disodium salt) were from Sigma. The ion exchangers were prewashed with aqueous NaCl and H_2O or 0.1*M* NaOH in 50% ethanol, followed by H_2O, 0.1*M* HCl, and H_2O. Formamide was purified by treatment with Amberlite MB-1 ion-exchange resin.[17] Polynucleotide concentrations are expressed in molarity of phosphate. Semicarbazide solutions were freshly prepared using the hydrochloride salt.

Semicarbazide Modification of CDP

To 200 μL of 2*M* semicarbazide–1*M* sodium bisulfite (pH 4.8) was added approximately 25 μmol of CDP (disodium salt). The solution was incubated at 37°C. After 2.5 h, 1 mL of 95% ethanol was added and the mixture was chilled in ice water. The precipitate was isolated by centrifugation and dissolved in 20 mL of H_2O. The solution was incubated at 37°C for 1 day to allow elimination of HSO_3^-.[18] It was then passed through a 0.7 × 26-cm column of DEAE cellulose (Cl^-). The column was washed with H_2O and then 20 mL of 0.1*M* triethylammonium bicarbonate. The product (compound 1) was eluted with 20 mL of 0.5*M* triethylammonium bicarbonate and the solution was lyophilized. The residue was dissolved in H_2O and lyophilized again. The residue was redissolved in water and a portion

was diluted with buffer and the uv-absorption spectrum was recorded. The yield, calculated using the published[18,19] ϵ_{275} of the cytidine analog (10^4), was approximately 70%. Semicarbazide modification was judged to be successful on the basis of the enhanced and red-shifted uv absorption in alkaline solution and the formation of uridine upon acid hydrolysis.[19]

Azo Derivative of CDP

To 10.2 mL of a 0.066 m*M* u⁴CDP solution (50 m*M* NaH_2PO_4, pH 7) chilled in ice water was added 50 μL of a cold 28 m*M* solution of *N*-bromosuccinimide in 50 m*M* neutral phosphate buffer. The uv-absorption spectrum was recorded immediately. The ^{1}H-nmr spectrum of a similarly oxidized sample in ^2H_2O revealed δ 6.97 [d, J = 7 Hz, C(5)H] and 8.92 [d, J = 7 Hz, C(6)H].

Methacrylate Polymerization by the Azo Pyrimidine Nucleotide

Polymerization of 1-glyceryl methacrylate (2,3-dihydroxypropyl methacrylate) was typically carried out as follows. To 30 μL of approximately 320 m*M* u⁴CDP in 0.05*M* neutral phosphate buffer in an ice bath was added 356 μL of a cold 54 m*M* aqueous solution of *N*-bromosuccinimide. The solution turned bright yellow immediately, indicating formation of the azo nucleotide (compound 2). An aliquot (75 μL) was added to a mixture of 0.26 g of 1-glyceryl methacrylate and 0.38 mL of 0.7*M* sodium acetate (pH 3.3). The solution (final pH 3.7) was covered and allowed to stand in the dark. After standing overnight it was found to have set to a colorless gel. Control solutions with *N*-bromosuccinimide or succinimide (but no u⁴CDP) remained liquid.

Fluorescent Labeling of the Methacrylate Polymer

To achieve fluorescence labeling, a thin slice (approximately 2 mm) of the glycol-bearing polymer, poly(glyceryl methacrylate), was treated with 1% $NaIO_4$ for 15 min to generate formaldehyde and polymer-bound aldehyde groups. Formaldehyde was removed by washing the gel slice in water for 1 h. At this point a portion of the polymer gave a positive test with Schiff's reagent. A slice that had not been treated with $NaIO_4$ gave a negative test, as expected. To attach fluorescent markers to the polymer via the polymer-bound aldehyde groups, the $NaIO_4$-oxidized gel was treated with 5 m*M* dansylhydrazine in 2-methoxyethanol for 10 min. The sample was then washed in water for 1 h. Examination under long-wavelength uv illumination revealed bright yellow-green fluorescence. A control slice of gel (i.e., no $NaIO_4$ treatment) did not appear fluorescent when treated similarly.

Enzymatic Addition of Modified Nucleotides to RNA

Semicarbazide-modified nucleotides were added enzymatically to oligo(A) primers. This was accomplished using the modified nucleoside diphosphate (compound 1), "primer-dependent" polynucleotide phosphorylase, and a primer. Oligo(A) primers were selected because of their resemblance to the 3′-poly(A) tail of the mRNA molecules that will be future probes. In this study the primers used were ApApA, $(Ap)_9A$, and [5′-^{32}P]-$(pA)_{10}$. A typical reaction mixture was prepared as follows. To 50 μL of 12.5 m*M* $MgCl_2$, 0.5 m*M* Na_2EDTA, 125 m*M* Tris-HCl (added from a 0.5*M* stock solution, pH 8.2), 0.25 mg/mL bovine serum albumin, 25 m*M* u^{4}CDP, and ca. 7.5 units/mL "primer-dependent" polynucleotide phosphorylase (primer dependent/nondependent = 15), was added 10 μL of water or 10 μL of 290 A_{260} units/mL $(Ap)_9A$ solution. The solutions were kept at room temperature.

The spectral characteristics of the polynucleotide product were studied in the case of the ApApA-primed reaction. After 24 h, the reaction mixture was passed through a DEAE Sephadex (Cl^-) ion-exchange column. Washing with water and then 0.2*M* NaCl was carried out to remove u^{4}CDP and then the polynucleotide was eluted with 1*M* NaCl. After addition of buffer (pH 7), the uv-absorption spectrum was recorded. The solution was then chilled, and an aliquot of a freshly prepared solution of *N*-bromosuccinimide was added. The uv-absorption spectrum was recorded immediately.

To follow the kinetics of polynucleotide formation, aliquots (5 μL) were periodically removed and subjected to thin-layer chromatography on cellulose plates. The developing solvent was 95% EtOH/1*M* NH_4OAc (11:9, v/v). This system has previously been used for isolating the polynucleotide product in polynucleotide phosphorylase reactions.[20] The polynucleotide remained at the origin, and after development, the band at the origin was scraped off the plate. The product was eluted by soaking the cellulose in 1 mL of H_2O. The u^{4}CDP band was treated similarly. The extent of polynucleotide formation was estimated by uv-absorption spectroscopy.

To assure that primer was indeed being utilized, ^{32}P-labeled primer was included in a reaction mixture, and the mixture was subjected to polyacrylamide gel electrophoresis (12% gel/7*M* urea)[21] and autoradiography. In these experiments the primer used was [5′-^{32}P]-$(pA)_{10}$, which was prepared from $(Ap)_9A$ by treatment with polynucleotide kinase and [γ-^{32}P]-ATP. Yeast $tRNA^{Phe}$ was run as a length standard.

Preparation and Properties of Poly(u^{4}C)

To 4.5 mL of 2.2*M* semicarbazide–1.1*M* $NaHSO_3$–5.5*M* urea (pH 5.0) was added 0.5 mL of 25 m*M* poly(C). To dissolve all components, the semicarbazide solution was carefully prepared from semicarbazide-HCl, Na_2SO_3, urea, and aqueous NaOH. The poly(C) solution was incubated at 37°C for 1.3 h and then dialyzed against H_2O for 3.5 days. The solution

was adjusted to neutral pH and its uv-absorption spectrum was recorded. Nucleotide analysis was performed by acid hydrolysis and cation-exchange chromatography.[22]

Since poly(C) forms a double helix with poly(I), the possibility that poly(u^4C) might do likewise was explored. Equimolar solutions of poly(u^4C) and poly(I) at approximately 25°C were mixed (0.1*M* NaH_2PO_4, pH 7.0, 0.1*M* NaCl) in different ratios[23] and the absorbance of the resulting solutions was measured at 248 nm. For comparison, unmodified poly(C) was mixed with poly(I) in different ratios and the absorbances were likewise measured.

Oxidation of Poly(u^4C)

Conversion of poly(u^4C) into the corresponding azo derivative was typically carried out as follows. To 0.5 mL of aqueous poly(u^4C) was added 25 μL of 0.2*M* NaH_2PO_4 (pH 7.0) and the resulting solution was chilled in ice water. One to two molar equivalents of *N*-bromosuccinimide were then added using a cold, freshly prepared aqueous solution.

Heat Stability of Poly(u^4C)

A mixture of 0.12 mL of 1.5*M* NaCl–0.15*M* sodium citrate (pH 7) and 0.30 mL of formamide was equilibrated at 65°C. To this mixture was added 0.18 mL of either H_2O or aqueous poly(u^4C). After 1 h at 65°C, the solution was incubated at 37°C for 24 h. The solution was cooled and passed through a 0.7 × 2.6 cm DEAE Sephadex A-25 column. The column was washed with 10 mL of H_2O, and the polynucleotide was eluted with 1*M* NaCl (final volume 5.0 mL). Portions of the eluate were subjected to phosphate analysis and oxidation with 2 molar equivalents of *N*-bromosuccinimide (2 min at 0°C, neutral buffer). For comparison, the original, unheated poly(u^4C) solution was analyzed for phosphate and similarly oxidized (after diluting into 1*M* NaCl).

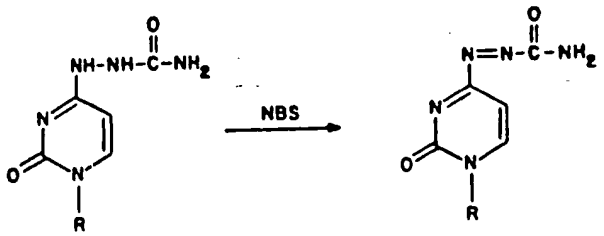
Decomposition of AzoRNA in Acid

To 2.2 mL of azoRNA [prepared from poly(C) by the semicarbazide method described above] dissolved in 25 m*M* NaH_2PO_4 (pH 7.0) was added 150 μL of 0.1*M* HCl. The uv-absorption spectrum was recorded before the addition and periodically after the addition of acid.

RESULTS

Azo Nucleotides and Polynucleotides

Bisulfite-catalyzed modification of CDP by semicarbazide produced the corresponding N^4-ureidocytosine nucleoside diphosphate (compound 1; see Fig. 1). The uv-absorption spectrum of compound 1, shown in Fig. 2



1 2

Fig. 1. N^4-Ureidocytosine (1) and 4-aminocarbonylazo-2-pyrimidinone (2) derivatives; R, ribofuranosyl 5′-diphosphate, NBS, *N*-bromosuccinimide.

(curve A), is very similar to several other semicarbazide-modified cytosine compounds with different groups attached to the N(1) position.[16,22] Treatment of modified CDP with *N*-bromosuccinimide in neutral buffer at 0°C produced the corresponding azo compound, compound 2. The uv-absorption spectrum reflected this transformation by manifesting a broad peak at approx. 350 nm (Fig. 2, curve B), as found in the spectra of

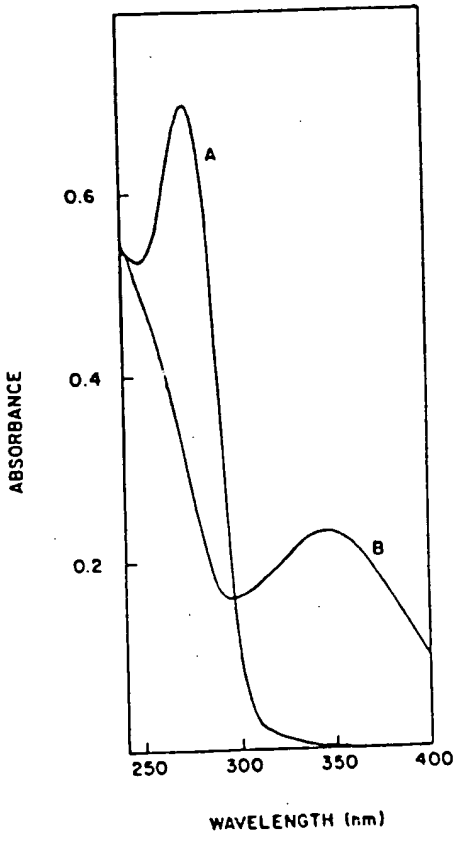


Fig. 2. The uv-absorption spectra of compounds 1 (curve A) and 2 (curve B) in phosphate buffer at pH 7.

analogous azo pyrimidines that were characterized by uv and nmr spectroscopy and elemental analysis.[16]

Methacrylate Polymerization

Polymerization of 1-glyceryl methacrylate by the azo nucleotide (compound 2) in mild aqueous acid produced a clear, colorless gel. This polymeric product has pendant glycol groups for site-specific attachment of heavy atoms or fluorescent groups. Generation of polymer-bound aldehyde groups with $NaIO_4$ allowed coupling of dansylhydrazine, which produced a brightly fluorescent polymer.

Enzymatic Addition of Modified Nucleotides to RNA

The spectral characteristics of the polynucleotide produced from u^4CDP and ApApA by "primer-dependent" polynucleotide phosphorylase were studied. The presence of u^4C nucleotides in the product was indicated by the appearance of the characteristic peak at approx. 350 nm in the uv-absorption spectrum upon treatment with *N*-bromosuccinimide.

The kinetics of polynucleotide formation in the presence and absence of the primer $(Ap)_9A$ are shown in Fig. 3. In the presence of primer (circles), polynucleotide formation was faster than in the absence of primer (squares), implying that the primer was being utilized. Conversion of nucleoside diphosphate into polynucleotide, estimated on the basis of the absorbance increase, occurred to the extent of approx. 40% in 24 h.

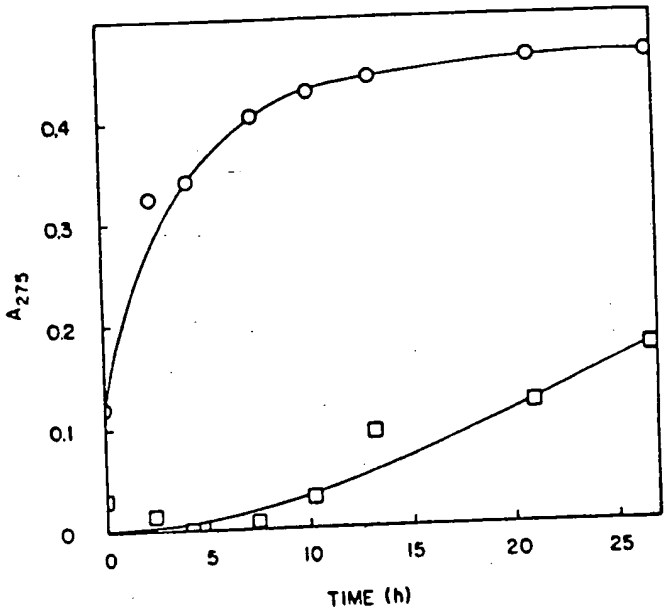


Fig. 3. Kinetics of polynucleotide formation by polynucleotide phosphorylase in the presence (circles) and absence (squares) of $(Ap)_9A$ primer. The absorbance at time zero in the primed case is due to $(Ap)_9A$.

To verify that primer was being utilized, [5′-^{32}P]-$(pA)_{10}$ was included in the reaction mixture, and polyacrylamide gel electrophoresis (12% gel/7*M* urea) was carried out. Under these conditions, natural and synthetic polyribonucleotides and polydeoxyribonucleotides migrate on the basis of chain length (i.e., number of nucleotides).[21] The autoradiogram of such a gel is shown in Fig. 4, as is a photograph taken under uv illumination after ethidium bromide staining to reveal yeast $tRNA^{Phe}$, a size marker (76 nucleotides). The results clearly show that radiolabeled material of length greater than 76 nucleotides is present. Thus, primer was utilized by the polynucleotide phosphorylase to produce [5′-^{32}P]-$(pA)_{10}(pu^4C)_n$.

Poly(u^4C)

Treatment of poly(C) with semicarbazide-bisulfite in urea resulted in modification of the cytosine nucleotides. The progress of the reaction was monitored by uv-absorption spectroscopy.[18] When the reaction was complete, the solution was dialyzed, during which time HSO_3^- was eliminated. This produced a polynucleotide with N^4-ureidocytosine nucleotides, poly(u^4C). The fraction of nucleotides modified was judged to be greater than 85% on the basis of hydrolysis and nucleotide analysis. As expected, treatment of poly(u^4C) with *N*-bromosuccinimide in neutral buffer at 0°C produced a polynucleotide containing azo nucleotides. The

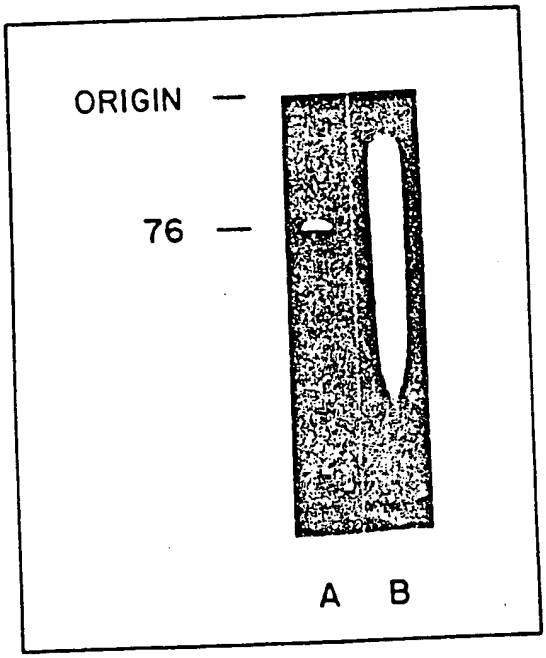


Fig. 4. Polyacrylamide gel electrophoresis of [5′-^{32}P]-$(pA)_{10}(pu^4C)_n$. The enzymatically synthesized RNA is shown in B, and yeast $tRNA^{Phe}$ is shown as a size marker (76 nucleotides) in A. Both samples were run on the same denaturing gel, which was stained with ethidium bromide and photographed with uv illumination for A and then dried and covered with film to obtain the autoradiogram shown in B. See text for experimental details.

uv absorption spectrum displayed the expected broad peak at approx. 350 nm.

Mixing buffered saline solutions of poly(u^{4}C) and poly(I) in different ratios resulted in no detectable hypochromicity, as shown in Fig. 5 (open circles). Since poly(C) and poly(I) combine to form a double helix with concomitant hypochromicity (see Fig. 5, solid circles), it appears that poly(u^{4}C) does not form a double helix with poly(I) under these conditions.

Heat Stability of Poly(u^{4}C)

Poly(u^{4}C) prepared by semicarbazide modification of poly(C) was heated in aqueous formamide under typical *in situ* hybridization conditions.[8,10–12,14] After an initial short period of high-temperature (65°C, 1 h) treatment for denaturing the chromosomal DNA molecule, a longer period at lower temperature (37°C, 24 h) is carried out to allow RNA·DNA annealing. To evaluate the extent of decomposition of azo precursor nucleotides caused by heating, samples of poly(u^{4}C) were oxidized with NBS before and after heating. It was found that the ϵ_{350} of the oxidized, unheated polymer was 2.91×10^3, whereas after heating the oxidation revealed that ϵ_{350} had dropped to 2.75×10^3. Thus, 95% of the azo precursor nucleotides survived the heat treatment. The above results are based on an 83% recovery of polynucleotide after heating.

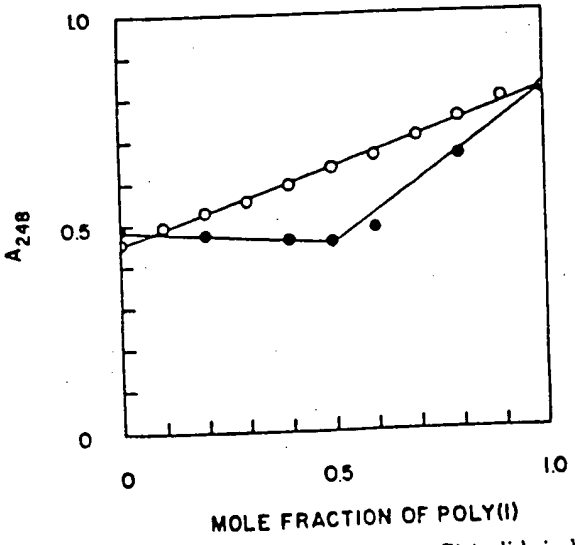


Fig. 5. The uv-absorption of mixtures of poly(I) and poly(C) (solid circles) or poly(I) and poly(u^{4}C) (open circles). Formation of poly(I)·poly(C) is evidenced by hypochromicity (absorbance minimum at 0.5 mol fraction), but no evidence for poly(I)·poly(u^{4}C) formation is apparent.

Decomposition of AzoRNA in Acid

Treatment of azoRNA with aqueous acid resulted in the disappearance of the peak at approx. 350 nm in the uv-absorption spectrum, as shown in Fig. 6. We have previously shown[16] that when the azo pyrimidine monomer was treated with acid, it similarly lost the peak at 350 nm and initiated polymerization of a methacrylate designed to bind heavy atoms or fluorescent groups. Polymerization probably occurs by a free-radical mechanism.

DISCUSSION

We have prepared a cytosine ribonucleotide that may be suitable for increasing the sensitivity of the *in situ* hybridization method of gene localization. The nucleotide, enzymatically attached to RNA probes, is designed to initiate polymerization of a methacrylate monomer capable of binding heavy atoms or fluorescent groups. Since one initiator can cause the linking of many methacrylate monomer units,[16] methacrylate polymerization and marker attachment would replace the radioactive decay and photographic development steps of the conventional *in situ* hybridization technique.

The nucleotides studied were semicarbazide-modified CDP (u^4CDP; Fig.

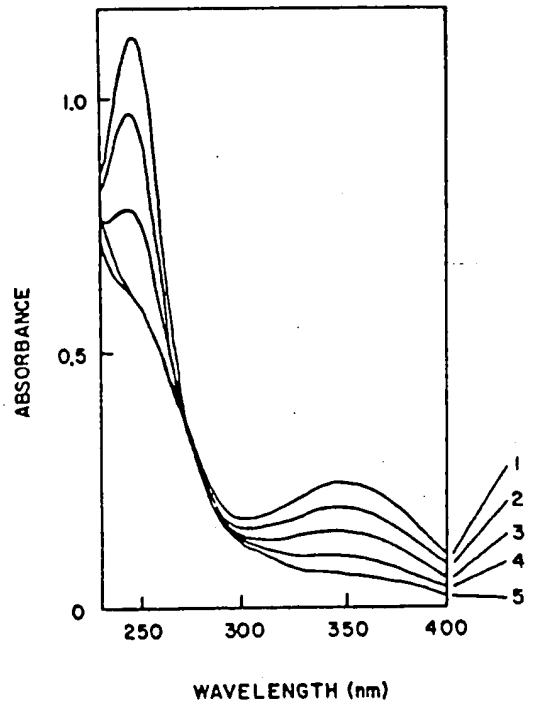


Fig. 6. Decomposition of azoRNA in acid. The uv absorption of *N*-bromosuccinimide-oxidized poly(u^4C) was recorded before addition of acid (curve 1) and at various times after addition of acid: 1 min (curve 2), 0.7 h (curve 3), 2.3 h (curve 4), and 4.5 h (curve 5).

1, compound 1) and its oxidation product, the azo pyrimidine nucleotide (Fig. 1, compound 2). Although the azo pyrimidine is the substance whose decomposition triggers methacrylate polymerization,[16] direct enzymatic synthesis of the probe mRNA$(A)_m$(azo nucleotide)$_n$ from compound 2 and poly$(A)^+$ mRNA would be precluded by the instability of compound 2 in solution. Thus, compound 1 must be used in the enzymatic synthesis of mRNA$(A)_m(u^4C)_n$, which in turn must be oxidized to mRNA$(A)_m$(azo nucleotide)$_n$. Addition of the u^4C nucleotides to oligo(A) and their subsequent oxidation were studied as a model for the analogous transformations to be carried out on the 3′-poly(A) tail of mRNA molecules of interest.

Preparation of compound 1 was achieved by treatment of CDP with semicarbazide-bisulfite followed by elimination of bisulfite.[18] Upon treatment of the N^4-ureidocytosine nucleotide (compound 1) with *N*-bromosuccinimide, the azo pyrimidine nucleotide (compound 2) formed (Fig. 1), as judged by the appearance of the characteristic peak[16] at approx. 350 nm in the uv-absorption spectrum (Fig. 2) and on the basis of its nmr spectrum. The azo nucleotide bleached and induced polymerization of 1-glyceryl methacrylate upon treatment with mild aqueous acid. This methacrylate was selected because it forms a polymeric hydrogel with pendant glycol groups. Cleavage of the glycol groups by $NaIO_4$ allowed the attachment of a fluorescent marker to the polymer.

Attachment of semicarbazide-modified nucleotides to the 3′-end of RNA primers was carried out using "primer-dependent" polynucleotide phosphorylase and the modified nucleoside 5′-diphosphate (compound 1). With ApApA as primer, the polynucleotide product was isolated and its spectrum recorded. Verification that u^4C nucleotides had been incorporated into the polynucleotide was obtained by *N*-bromosuccinimide oxidation of the polynucleotide product. Oxidation produced the characteristic peak at approximately 350 nm in the uv-absorption spectrum.

The kinetics of polynucleotide formation were followed using $(Ap)_9A$ as primer. Conversion of u^4CDP into polymer-bound u^4C nucleotides reached approx. 40% in 24 h and had the kinetics shown in Fig. 3 (circles). The unprimed reaction was found to be much slower (Fig. 3, squares). The difference in rates offered indirect evidence that $(Ap)_9A$ was being used as a primer for the reaction.

To assure that an oligo(A) would be used as a primer by the "primer-dependent" polynucleotide phosphorylase, [5′-^{32}P]-$(pA)_{10}$ was used and the reaction product examined by polyacrylamide gel electrophoresis. Autoradiography revealed that the oligo(A) had indeed been used as a primer, and polynucleotides more than 76 nucleotides long had been formed (Fig. 4).

The *in situ* hybridization procedure typically requires that the probe RNA be heated in the presence of the chromosomal DNA.[8,10-12,14] To test the extent to which u^4C nucleotides incorporated into a polynucleotide might be lost by decomposition during heating, the following experiment

was performed. Poly(C) was converted to poly(u^4C) by treatment with semicarbazide-bisulfite and elimination of bisulfite. The extent of conversion of cytosine to u^4C nucleotides was judged to be greater than 85% by hydrolysis and nucleotide analysis. After heating at 65°C in buffered formamide for 1 h followed by 37°C for 24 h, the polynucleotide was treated with *N*-bromosuccinimide to generate the azo nucleotides. By measuring the A_{350} of the oxidized polynucleotide before and after heating, the loss of azo precursor was monitored. It was found that the heated polynucleotide produced 95% of the azo nucleotides that the unheated polynucleotide produced. Thus, the destruction of only 5% of the azo precursor nucleotides (u^4C) occurred.

The use of a probe molecule like mRNA$(A)_m(u^4C)_n$ for the hybridization step introduces the possibility that the probe will bind to DNA via the u^4C nucleotides. To assess the likelihood of such an event, the hybridization properties of poly(u^4C) were examined. Given that poly(C) binds to poly(I) and u^4C retains many structural similarities to unmodified C, the possible complexation of poly(I) with poly(u^4C) was studied. Solutions of poly(I) and poly(u^4C) were mixed in different ratios and the uv absorbance of the solutions was recorded. Figure 5 (open circles) shows that there is no evidence for the formation of a poly(I)·poly(u^4C) complex under these reaction conditions. For comparison, unmodified poly(C) was treated similarly, and the data support the conclusion that a 1:1 complex, poly(C)·poly(I), forms (Fig. 5, solid circles).[23] Whether DNA segments in the chromosome will be more effective than poly(I) for binding the modified nucleotides is unknown, and control experiments with actual specimens will have to be carried out. In any case, should such regions of the specimen exist, they ought to be identifiable by carrying out the *in situ* procedure with simple poly(u^4C). Thus, regions that hybridize to the mRNA portion of the probe mRNA$(A)_m(u^4C)_n$ ought to be distinguishable from regions that bind the $(u^4C)_n$ portion of the probe.

Ultimately, to generate the initiators of methacrylate polymerization, the specimen-bound probe must be treated with *N*-bromosuccinimide to convert the u^4C nucleotides into azo nucleotides. We previously showed[16] that u^4C nucleotides in DNA were acid labile, as is required for their usefulness as initiators of methacrylate polymerization. A similar test of the acid lability of u^4C nucleotides in RNA was carried out. Figure 6 shows that mild acid causes a disappearance of the peak at approx. 350 nm in the uv-absorption spectrum of the azoRNA. Such acid lability of the parent azo pyrimidine is associated with initiation of methacrylate polymerization.[16] The relatively mild acid required (pH 3.7)[16] is not expected to damage the fixed chromosomes prepared for microscopy. Thus, the azo pyrimidine nucleotides in the probe RNA molecule ought to function as initiators of methacrylate polymerization. Subsequent attachment of fluorescent groups or heavy atoms to the methacrylate polymer and visualization by microscopy may allow single-copy genes to be localized along eukaryotic chromosomes.

We thank Professor D. Lohr for assistance with the autoradiography experiments. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research, to the Arizona State University Research Council, and to NIH (Grant 1GM27886).

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Received February 27, 1981
Accepted May 5, 1981